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(From the Bacteriological Department of the Institute of Tropical Hygiene and the Bacteriological Laboratory of the Municipal Public Health Service, Amsterdam).

RESEARCHES ON LEPTOSPIROSIS BALLUM

The detection of urinary carriers in laboratory mice

by

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(Received January 22, 1949).

The following investigations on the incidence and behaviour of *Leptospira ballum* in laboratory mice were prompted by an accidental human infection (which befell one of us) with this type of Leptospira. A clinical description of the case has already been published (3). As this is probably the first human infection with *L. ballum*, it might be of interest to report the history.

1. The human infection.

The illness started acute, with high fever and severe headache. In following days the fever took a continuous course between 37.8 and 38.8° C. with nightly peaks up to 40° C. Some joints became painful on the third day and gradually other joints were attacked (except the mandibular articulation). The skin above the painful joints felt warm but no redness or swelling was observed. Muscular pain was entirely absent. On the same day the eyes showed a very slight conjunctival injection; the next day herpes labialis was noticed. There was no jaundice or rigidness of the neck but the patient suffered from severe dizziness. Lytic abatement of fever started after five days; the headache subsided only gradually during convalescence (which took about three weeks).

Physical examination revealed no abnormalities; the spleen, liver and lymph nodes were not enlarged. Extensive examination of blood, faeces and urine was unhelpful for diagnosis. Special investigations for influenza and psittacosis virus or for infection with brucella or bacilli of the typhoid group were all negative.

However, agglutination of the patient's serum with different strains of leptospiras, repeated after convalescence produced the following interesting results.

Table I.

Agglutination titres in patient's serum with different strains of leptospiras.

Leptospira strain	serological type	titre on day after beginning of illness				
		day				
		4	40	43	62	340
Kantorowicz	icterohaemorrhagiae biotype A	0	30		30	0
Wijnberg	icterohaemorrhagiae biotype AB	0	0		0	0
Hond Utrecht IV	canicola	0	10		30	0
Andaman CH 31	grippotyphosa	0	0		0	0
Mallersdorff II	sejrö	0	0		0	0
Pomona	pomona	0	0		0	0
S-102	ballum	10	1000	>1000	>1000	300
SR	ballum				3000	
Saxkoebing	saxkoebing			100		
Sarmin	sarmin		0			
Swart van Tienen	bataviae			0		
Paidjan	paidjan			0		
Rachmat	autumnalis biotype A			0		
Akiyami A	autumnalis biotype AB			0		
Bankinang	bankinang			0		
Sentot	sentot			0		
Salinem	pyrogenes			0		
Zanoni	australis B			0		
Ballico	australis A			0		
Hebdomadis	hebdomadis			0		
Hond HC	hond HC			10		
3705	3705			0		
Hardjopratjitno	hardjopratjitno			0		
Djassiman	djassiman			0		
Naam	naam			0		
Cynopterus 3868	Cynopterus 3868			0		
90 C	90 C			0		
Rat Semarang 173	rat Semarang 173			0		
Veldrat Batavia 46	javanica			0		
Andaman CH 11	andaman CH 11			300		

The high positive titre for the two strains of *L. ballum* which had developed from 1:10 to 1:3.000 during the course of the disease pointed to a leptospiral infection.

When a search was made for a possible aetiological moment, the patient remembered having handled one of a batch of laboratory mice about nine days before the onset of the illness at a time when a strain of rat-bite fever spirilla, which was kept in these mice, was transferred to two other mice. During the operation the mouse had superficially scratched the finger of the patient without making a visible scar. The scratch was nevertheless immediately treated with aethyl spirit.

Fortunately, the mouse could still be traced and proved to be a urinary carrier of leptospiras. The mouse's bloodserum with a strain of *L. ballum* type gave an agglutination titre of 1:300. The mouse was then killed and a strain of leptospira isolated from the kidneys (strain *SR*); serologically it proved to be identical to the strains *mus 127* and *S-102*, described in the next chapter. Furthermore, this strain *SR* was agglutinated by the patient's serum (62nd day) to a titre of 1:3.000.

The origin of the human infection with *L. ballum* was now clear. Unfortunately the diagnosis was made only after convalescence, and it was then too late to try and isolate a leptospira strain out of the patient's blood. On the 4th day serum only (by some mistake) had been sent to the leptospira laboratory. Repeated examinations of the patient's urine remained negative for leptospira; inoculation of the urine of the 44th day in 4 mice and 3 guinea pigs gave no results.

2. The isolation and serological pattern of various strains of *Leptospira ballum*.

The type species of this leptospira strain (*mus 127*) was isolated by Borg Petersen (2) in 1943 out of a rural house mouse — *Mus musculus spicilegus* — caught on a farm near Ballum in Southern Jutland, Denmark. The strain was serologically distinct from all other types as tested by Borg Petersen.

Schüffner and Bohlander, however, had in 1941 detected leptospiras in the peritoneal fluid and urine of a white mouse, which had been experimentally inoculated with Spir. minus [1]). A week

[1]) This was another strain of spirilla than the one with which the patient worked.

later a second mouse of the same batch showed leptospiras in the urine. Blood from the first mouse was inoculated into VERVOORT's

TABLE II.

Serological reactions of *Leptospira ballum* strains with different anti-sera.

Anti sera (titre with homologous strain 1 : 10.000)	serological type	*Leptospira ballum* strains				
		S-102	Mus 127	Val-luère	SR	Iwema
Kantorowicz	icterohaemorrhagiae biotype A	100	100	30	300	300
Wijnberg	icterohaemorrhagiae biotype AB	>100	0	0	30	30
Hond Utrecht IV	canicola	100	>300	>300	3000	1000
Andaman CH 31	grippotyphosa	0	0	0	0	0
Sejrö M-84	sejrö	0	10	30	30	30
Pomona	pomona	0	0	0	0	0
S-102	ballum	10.000	30.000	10.000	30.000	30.000
Saxkoebing	saxkoebing	0	0	0	0	0
Sarmin	sarmin	300	100	100	300	>100
Swart van Tienen	bataviae	0	0	0	0	0
Paidjan	paidjan	0	0	0	0	0
Rachmat	autumnalis biotype A	0	0	0	0	0
Akiyami A	autumnalis biotype AB	0	0	0	0	0
Bankinang	bankinang	0	0		0	
Sentot	sentot	0	0			
Salinem	pyrogenes	30	0	30	100	100
Zanoni	australis B	30	0			
Ballico	australis A	0	0	0	0	0
Hebdomadis	hebdomadis	0	0	0	0	0
Hond HC	hond HC	0				
3705	3705	0		0	0	0
Hardjopratjitno	hardjopratjitno	0				
Djassiman	djassiman	0	0	0	0	0
Naam	naam	0	0		0	0
Cynopterus 3868	cynopterus 3868	0	0		0	0
90 C	90 C	0	0		0	0
Rat Semarang 173	rat Semarang 173	0	0		0	0
Veldrat Batavia 46	javanica	0	0	10	0	0
Andaman CH 11	andaman CH 11	0	0	0	0	0
3885	3885	0				
Mankarso	mankarso	100	10			
Benjamin	benjamin	0				0

TABLE III.

Agglutination reactions of different leptospira strains with S-102 anti-serum.

Leptospira strain	serological type	anti-serum S-102
Kantorowicz	icterohaemorrhagiae biotype A	20
Wijnberg	icterohaemorrhagiae biotype AB	10
Hond Utrecht IV	canicola	100
Andaman CH 31	grippotyphosa	0
Mallersdorff II	sejrö	0
Pomona	pomona	0
S-102	ballum	10.000
SR	ballum	30.000
Iwema	ballum	10.000
Ballum M 127	ballum	30.000
Valluère	ballum	10.000
Saxkoebing	saxkoebing	0
Sarmin	sarmin	100
Swart van Tienen	bataviae	0
Paidjan	paidjan	0
Rachmat	autumnalis biotype A	0
Akiyami A	autumnalis biotype AB	0
Bankinang	bankinang	0
Sentot	sentot	0
Salinem	pyrogenes	0
Zanoni	australis B	30
Ballico	australis A	0
Hebdomadis	hebdomadis	0
Hond HC	hond HC	0
3705	3705	0
Hardjopratjitno	hardjopratjitno	0
Djassiman	djassiman	0
Naam	naam	0
Cynopterus 3868	cynopterus 3868	0
90 C	90 C	0
Rat Semarang 173	rat Semarang 173	0
Veldrat Batavia 46	javanica	0
Andaman CH 11	andaman CH 11	0
3885	3885	0
Mankarso	mankarso	0
Benjamin	benjamin	0
Poi	poi	0
Pie	pie	0
Sari	sari	0
Bovine Olitzki	bovine Olitzki	0
Mitis Johnson	mitis Johnson	0

medium and a strain of leptospira was isolated (S-102) which proved to be serologically different from all other type strains then in possession of the SCHÜFFNER Laboratory at the Institute of Tropical Hygiene in Amsterdam. Owing to war time conditions and shortness of laboratory animals only a few experiments could be made with strain S-102 [1]).

Some time later an exchange was made with BORG PETERSEN's strains of *L. ballum* (mus 127 and Valluère). Strain S-102 then proved to be serologically identical to the type-strain of *L. ballum* and our newly isolated strain SR. Finally a 4th strain of the same type (strain *Iwema*) was isolated out of another mouse colony (see next chapter).

The serological reactions, performed with these strains, are presented in Tables 2 and 3.

Reciprocal absorption tests with the various strains of *L. ballum* and anti-sera have been carried out in part but are not yet finished. A preliminary test performed with antiserum of S-102 and strain Valluère gave complete absorption with this strain.

3. THE NATURAL OCCURENCE OF *Leptospira ballum* IN LABORATORY MICE.

The detection of leptospiras of type *ballum* in the mouse that caused the human infection led to the search for leptospiras in the urine of other batches of white mice. The results of these investigations can be summarized as follows:

Of 30 apparently healthy adult mice from the stock breeding in the Municipal Bacteriological Laboratory in Amsterdam, 28 mice showed leptospiras in the urine, sometimes in large numbers. Of 15 young mice (weight under 10 grammes) only one was positive.

However, after isolation the young ones all became positive. The excretion of leptospiras was rather irregular; on some days the urine teemed with leptospiras — about as much as in a well-grown culture — while on other days only a few could be detected. During an observation period of more than 12 months the urine remained positive without impairing the health of the mice; they thrived, and bred normally. A strain isolated out of the kidney of one of the mice was serologically identical to the type strains of *L. ballum*.

The two adult negative mice were observed for a period of one

[1]) For the same reason, these investigations were not yet published.

month, but the repeatedly examined urine remained negative for leptospiras. The mice were then killed; agglutination of the blood serum was positive for *Leptospira ballum* and a leptospira strain of type ballum was isolated out of the kidneys.

It was not possible to trace the origin of the infection in this mouse colony. The breeding had been started from two white mice out of SCHÜFFNER's Laboratory, and this stock of mice was lost during the war.

A number of positive mice from the colony in the Municipal Laboratory were kept in the laboratory for further experiments while the remaining positives were killed.

Examination of some newly acquired mice, housed in the laboratory of Tropical Hygiene revealed a second positive urine carrier. This mouse, among others, was bought from an amateur dealer in Amsterdam. He gave full cooperation when asked if we could examine his stock. From a sample of 80 mice out of about 200 in his possession, 63 were positive, while again a strain cultured out of the kidneys of an urinary carrier (strain *Iwema*) proved serologically identical to the strains mus 127, S-102 and SR. The dealer started to breed a mouse colony in 1946 from a few couples of white mice bought from schoolboys. No further investigations on the origin of this infection could therefore be made. A number of mice from the dealer's stock were retained for further experiments; the remainder were killed and the dealer has now started to breed a colony from mice found free of leptospiras.

A search for leptospira in the urine of mice from other sources was entirely negative as is shown in table 4.

TABLE IV.

Leptospira in mice from different breeding stock.

Origin	number examined	number with leptospiras in urine
Municipal laboratory	adult 30	28
	young 15	1
Inst. Trop. Hyg. (own breeding)	18	0
Mouse dealer	80	63
Hyg. lab. Utrecht	25	0
Chinine factory	28	0
Lab. cancer research	56	0

Leptospiras of type *ballum* have thus been found in three different mouse colonies, one in 1941, the two others recently. It may of course be possible that as the positive findings were all made in Amsterdam, all three colonies had a common origin, but this cannot be confirmed. We have also given thought to the possibility that the infection could have been introduced incidentally by wild mice which at one time or an other had mixed with the white mouse colonies. Examination of mice (*Mus musculus*) caught at the Amsterdam breeder's stable and in the Municipal Laboratory have so far been negative.

Besides in Southern Denmark the carrier of *Leptospira ballum* — *Mus musculus spicilegus* — is found in Southern and Eastern Europe, but never in the Netherlands. Other reports on the natural occurrence of leptospira in mice are scarce. In the early period of leptospira research BESSEMANS and THIRY (1) described the occurrence of leptospiras in one of their colonies of laboratory mice at Ghent while a stock of mice in the histological laboratory at Ghent also was infected. As these mice were fed with bread soaked in tap water, the authors were of the opinion that the isolated strain of leptospiras were saprophytic water leptospiras from the main water supply; however, after feeding the animals with bread moistened with sterile water, positive urines were still detected.

Experiments with this strain were made the following year by DINGER and VERSCHAFFELT (4) in the Institute of Tropical Hygiene at Amsterdam. They received from BESSEMANS four mice from his stock of laboratory mice, the former were urinary carriers of leptospira, and out of the urine a strain of leptospiras was isolated (*"Muis Gent"*).

This strain proved to be serologically different from BESSEMANS' strain of water leptospira, and did not show any affinity to different strains of pathogenic leptospiras from European and Asiatic sources known at that time. Furthermore, the authors investigated the urine of a number of white mice in the Amsterdam laboratory; one hundred examinations were all negative.

The strains of BESSEMANS in Ghent and *"Muis Gent"* in Amsterdam were unfortunately lost many years ago and no further typing with ballum antisera is possible.

4. A SEARCH FOR OTHER POSSIBLE HUMAN INFECTIONS WITH *L. ballum*.

No reports on other human infections have been found in lite-

rature. Since 1944, BORG PETERSEN has agglutinated all samples of sera sent to the Statens Serum Institute in Copenhagen with the ballum strain and has never found a significant major ballum titre [1]). Co-agglutination reactions were observed in some infections with *L. canicola* and *L. sejrö*. Since October 1945, we have done the same with our S-102 strain and have likewise never found a specific agglutination before the laboratory infection took place.

Altogether 1.000 agglutination reactions of the S-102 strain with samples of human sera have been carried out by us. Non-specific agglutinations, nearly always to a lower titre than the specific agglutinations, were encountered 68 times in 44 cases of leptospirosis. Of these, 20 were caused by *L. icterohaemorrhagiae* and 24 by *L. canicola*. As will be seen in Table 5, co-agglutinations with strain S-102 were observed more often and in higher titre in *L. canicola* than in *L. icterohaemorrhagiae* infections, a fact which MINKENHOF also has mentioned in his paper on leptospirosis canicolaris (5). This agrees with the fact that both ballum- and S-102-strains seem to possess an antigenic factor in common with the canicola strain (1 percent of the homologous titre) as was revealed by reciproque agglutination reactions with their antisera (see Tables 2 and 3).

TABLE V.

Non-specific leptospira S-102 agglutination reactions in cases of Weil's disease and leptospirosis canicolaris.

S-102 titre expressed as percentage of highest homologous titre	Number of cases	
	Weil's disease	febris canicolaris [2])
0.3	3	2
1	6	5
3	8	4
10	2	6
30	1	6
100	0	1
	20	24

[1]) Personal communication in May 1948.

[2]) Included are the agglutination reactions reported by MINKENHOF; these reactions were carried out in the bacteriological laboratory of the Institute of Tropical Hygiene.

Finally, we performed a number of agglutination reactions with strains of *L. ballum* in sera of six persons who had been in contact with the positive mouse colonies. The results were entirely negative.

5. Laboratory investigations with the type strains of *L. ballum*.

A. Virulence for mice.

For mice the virulence of the strains of *L. ballum* is nil or very low. As Borg Petersen found with his strains, inoculation of our strains S-102, SR or Iwema in mice did not make the animals ill, although during the first days leptospiras could invariably be found in the peritoneal fluid and in the blood. But in the long run practically all infected animals became urinary carriers. This carrier state seems to remain during the whole life span of the mouse without impairing its health.

Contact infections seem to take place regularly, but it takes from one to three months before negative contacts become positive; this was observed by placing healthy negative mice in a cage together with one or more positive animals. Young mice from a positive mother that remained in close contact with the mother were all negative during the first weeks but became urinary carriers in the course of months.

However, when *L. ballum*-positive blood, peritoneal fluid or kidney suspensions are inoculated intraperitoneally in white mice, the urinary carrier state is attained much more quickly. Again, the animals remained alive and healthy after inoculation with strains S-102 or SR and leptospiras were first detected in the urine on the eighth day after infection up till the 19th day (different experiments). These mice thereafter remained urinary carriers during life time.

We also tried to ascertain whether mice from a *L. ballum* infected colony were more resistant to infection with virulent *L. icterohaemorrhagiae*.

Batches of mice of different origins (adult urinary carriers, young mice out of litters from positive and negative mothers in a ballum-infected colony) were inoculated intraperitoneally with 0.2 ml of a virulent strain from a human case of Weil's disease. The virulence was controlled by injecting the strain in a guinea-pig which died typically with jaundice and haemorrhages. As a further control a batch of healthy mice from a leptospira-negative colony was

inoculated at the same time. In total four batches of five mice were used in this experiment.

With a few exceptions the animals remained alive. Two mice died intercurrently from other causes. One young mouse of a litter from a negative mother of a ballum colony died on the tenth day after inoculation with *L. icterohaemorrhagiae*. Slight jaundice was noted, but no haemorrhages, no changes in liver, spleen and kidneys and no leptospiras were detected.

In the control batch of mice leptospiras were present in the peritoneal fluid on the fourth and fifth day after inoculation. Thereafter, no leptospiras could be found in peritoneal fluid nor in the blood, but on the 20th day leptospiras were detected in the urine of four of the mice. The fifth had died accidentally one week after inoculation; from a suspension of the kidneys a strain of *L. icterohaemorrhagiae* was isolated. Three months later the four mice were still alive and apparently healthy, but had remained urinary leptospira-carriers as shown by intermittent examinations.

The three batches of mice from a ballum-positive colony behaved in the same way as the control batch. A few days after inoculation the peritoneal fluid was positive while somewhat later, in a few mice of these batches leptospiras also were found in the blood during a period of one or two days.

Examination on the 20th day after inoculation revealed that all mice proved to be urinary leptospira-carriers and remained so thereafter.

Thus negative young mice, from a negative and from a positive mother out of a ballum-colony and adult *L. ballum* urinary carriers, showed no signs of immunity to infection with *L. icterohaemorrhagiae*.

The question arises whether the leptospiras present in the urine of the infected ballum-positive mice were of the ballum type or were derived from the inoculated strain of *L. icterohaemorrhagiae*. To elucidate this point, the following experiment was made.

Pooled urine of five ballum-positive mice, inoculated one month before with the *L. icterohaemorrhagiae* strain, was injected intraperitoneally into a guinea-pig, which was then used as a 'filter' for leptospira (SCHÜFFNER (6)). Fifteen minutes after inoculation, heart puncture of the guinea-pig was performed and the blood inoculated in two groups of tubes with VERVOORT's medium to which were added respectively anti-serum of *L. icterohaemorrhagiae*

and *L. ballum*, while another of the culture tubes contained only the medium. In this last tube a strain of leptospiras developed which was identical to the inoculated strain of *L. icterohaemorrhagiae* in the mice, whilst no growth occured in the other tubes. When, two days after inoculation of the guinea-pig, the peritoneal fluid contained many leptospiras, blood was again cultured and a second guinea-pig was inoculated. Further, in addition to the control without antiserum, the culture in the medium mixed with anti-S-102-serum also became positive; this strain proved to be *L. icterohaemorrhagiae*, whilst in the medium, mixed with anti-icterohaemorrhagiae-serum, growth was delayed, but three weeks later leptospiras of type icterohaemorrhagiae were present.

The second guinea-pig also died typically from the leptospiral infection. Thus it appears that the inoculated strain of leptospiras supplants the ballum type leptospira, originally present in the urine of the mice.

B. Virulence for guinea-pigs.

Borg Petersen states that the strains of *L. ballum* which he isolated were nearly as virulent for young guinea-pigs as *L. icterohaemorrhagiae*, although jaundice occurred less frequently in *L. ballum* infections and was not seen before the sixth passage. Our strains S-102 and SR seem to be less virulent; up to now we have not been successful in maintaining these strains in more than three guinea-pigs passages. The animal then recovered from the infection, and became a carrier. No jaundice occurred in the animals which died from the infection, and only slight haemorrhages were present.

Summary.

A case of an accidental human infection caused by *Leptospira ballum*, following a superficial scratch on a finger of the patient while handling a white mouse is described. This mouse proved to be a urinary carrier of *Leptospira ballum*.

Further investigations revealed that practically all mice of the same colony voided *Leptospira ballum* with the urine.

On examination of other mice-breeding colonies another colony infected with *L. ballum* was detected; in 1941 a strain which later also proved to be *L. ballum*, was isolated from the urine of a white mouse.

The various strains were analysed; they were serologically identical to Borg Petersen's type strain although the virulence for guinea-pigs was much less.

Mice from a *L. ballum*-positive colony proved to have no immunity to infection with virulent *L. icterohaemorrhagiae*, but — with one exception — remained alive and became carriers of *L. icterohaemorrhagiae*, whilst *L. ballum* could not be detected any more in the urine of these mice.

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SALMONELLA IN THE MESENTERIC LYMPH NODES OF HEALTHY PIGS

by

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(Received November 20, 1948).

In American and English literature the presence of *Salmonella* in mesenteric lymph nodes of healthy pigs has in later years been frequently reported. The following table in which the cases mentioned in TOPLEY and WILSON's handbook (1) are taken up offers a survey of the types recorded and the countries where these have been isolated.

TABLE I.

Type	Found in
S. paratyphi B	Uruguay
S. typhi murium	Mexico, Uruguay, U.S.A.
S. heidelberg	Mexico
S. chester	Uruguay
S. san-diego	Uruguay
S. reading	England
S. derby	Uruguay, Mexico, U.S.A.
S. bredeney	Uruguay
S. cholerae suis	South-America, Mexico, U.S.A., England
S. thompson	England
S. bareilly	U.S.A.
S. montevideo	Uruguay
S. newport	Uruguay, England
S. muenchen	Uruguay
S. oregon	U.S.A.
S. dublin	England
S. london	Uruguay, England
S. give	U.S.A.
S. anatum	U.S.A., Mexico
S. meleagridis	Mexico
S. newington	Uruguay, U.S.A.
S. senftenberg	Mexico
S. worthington	U.S.A.
S. cerro	Uruguay

In view of the importance that these findings might have for the epidemiology of human infection with *Salmonella* and for the meat control we investigated, whether also in this country *Salmonella* might occur in the mesenteric lymph nodes of healthy pigs. In this aim 503 slaughtered pigs were examined in which the inspection of neither the living nor the slaughtered animals had brought to light any anomalies. The following method was applied.

About 1 g of tissue of the lymph nodes is cut finely and introduced into a tube of tetrathionate broth modified according to KAUFFMANN. After incubation at 37° C. during about 20 hours a drop of this nutrient medium is streaked on a brilliant green agar plate which after an incubation at 37° C. during 20 hours is examined as to the growth of *Salmonella*.

In order to ascertain whether an infection of the mesenteric lymph nodes might be connected with the presence of *Salmonella* in the digestive tractus a small sample of feces of each of the pigs was examined as well.

The results of the above investigation was that in 14 out of 503 pigs examined (2.78 %) the presence of *Salmonella* in the mesenteric lymph nodes was established. From none of the samples of feces *Salmonella* could be isolated.

The strains of *Salmonella* isolated appeared to belong, to various types as reported in the following table.

TABLE II.

Salmonella-type	Antigenic formula	Number
S. typhi murium	IV, V, i : 1, 2	7
S. dublin	IX, g, p	4
S. enteritidis	IX, g, m	1
S. newport	VI, VIII, e, h : 1, 2	1
S. give	III, X, 1, v : 1, 7	1

The negative result of the examination of the feces does not preclude an infection of the lymph nodes from the intestinal tract. An extensive investigation into this matter and of the importance these findings might have for public health is carried on.

Summary.

Of 503 healthy pigs the mesenteric lymph nodes as well as the feces were examined as to the presence of *Salmonella*. In 14 animals (2.78%) the presence of these bacteria in the lymph nodes was established. The testing of all of the samples of feces gave negative results.

The following types of *Salmonella* were found: *S. typhi murium* (7 ×), *S. dublin* (4×), *S. enteritidis* (1 ×), *S. newport* (1 ×) and *S. give* (1 ×).

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(From the Serological Laboratory of the Office of the Chief Medical Examiner of New York City).

GENETICS AND NOMENCLATURE OF THE Rh-Hr BLOOD TYPES

by

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(Received February 1, 1949).

The Rh or rhesus factor obtained its name when the first antisera were prepared by LANDSTEINER and WIENER in 1937 by injecting into experimental animals blood of rhesus monkeys. At present, antisera of human origin are usually used, either from patients who have had intragroup hemolytic reactions, or from mothers of erythroblastotic infants, or from normal male individuals immunized by repeated injections of Rh-positive blood.

The human sera have the advantage of higher specificity and potency, so that less experience is required for their satisfactory use, thus explaining their greater popularity than animal anti-rhesus sera. While all the animal anti-rhesus sera produced to date have given identical reactions in parallel tests, three distinct varieties of Rh antisera have been obtained from human sources. Of the human Rh antisera, the one corresponding to the original anti-rhesus serum, reacting with the bloods of approximately 85 per cent of Caucasians, has been designated as anti-Rh_0. For the other two antisera have been selected the designations anti-rh′ for the one giving 70 per cent positive reactions, and anti-rh″ for the one giving 30 per cent positive reactions, to indicate that these two antisera are on an equal plane and to distinguish them from the more important anti-Rh_0 sera.

The three varieties of Rh antisera detect the presence or absence of three corresponding Rh factors in human blood, Rh_0, rh′, and rh″. Of these, Rh_0 is by far the most antigenic and therefore the most important clinically, as is indicated by the use of a capital "R". For solving most clinical problems, it is therefore sufficient

to make tests with standard anti-rhesus animal sera, or, preferably, with human anti-Rh_0 serum. This procedure is known as Rh testing, and with its aid individuals may be classified as either Rh-positive or Rh-negative (or, more strictly, Rh_0-positive and Rh_0-negative).

When tests are made with all three Rh antisera, the bloods of human beings can be subdivided into eight types; this procedure is called "Rh typing" to distinguish it from "Rh testing" or testing with anti-Rh_0 alone. This is not as complicated as it sounds, if the following principles are borne in mind. Anti-rh' and anti-rh'' are related like the agglutinins anti-A and anti-B, so in tests made only with anti-rh' and anti-rh'', four types are differentiated which are analogous serologically and genetically to the four blood groups. Since every individual is also either Rh_0-positive or Rh_0-negative, each of the four types may be further subdivided into two, and a double scheme of four types each results as shown in table I. Thus, the scheme of eight Rh types only amounts to a double blood group scheme, so that any one familiar with the four LANDSTEINER blood groups can quickly master the eight Rh types.

TABLE I.

Scheme of the eight Rh blood types.

Blood containing factor Rh_0 (Rh-positive)				Blood not containing factor Rh_0 (Rh-negative)			
Designation of types [1]	Reactions with sera			Designation of types	Reactions with sera		
	Anti rh'	Anti rh''	Anti Rh_0		Anti rh'	Anti rh''	Anti Rh_0
Rh_0	—	—	+	rh	—	—	—
Rh_1	+	—	+	rh'	+	—	—
Rh_2	—	+	+	rh''	—	+	—
Rh_1Rh_2	+	+	+	rh'rh''	+	+	—

[1]) Type Rh_1 contains the two factors Rh_0 and rh', the name Rh_1 being short for Rh'_0; similarly, Rh_2 is short for Rh''_0 and Rh_1Rh_2 is short for $Rh'_0Rh''_0$.

As for the names of the eight Rh types, these are merely determined by the antisera with which the blood reacts. Thus, type Rh_0 blood is so named because it reacts with anti-Rh_0 but not with anti-rh' or anti-rh'' sera; type rh' blood, on the other hand, reacts

with anti-rh′, but not with anti-Rh_0 or anti-rh″ sera; and so on. Blood not reacting with any of the three Rh antisera is simply designated type rh, or rh blood. This designation, besides being simple, has the advantage of avoiding ambiguity, since type rh is not identical with Rh-negative. The latter refers to blood giving a negative reaction when tested with standard anti-Rh (anti-Rh_0) serum alone. Blood which for clinical purposes is Rh-negative (Rh_0-negative) actually includes the four types rh, rh′, rh″, and rh′rh″ (see table I); *i.e.*, patients belonging to any of these four types should be considered Rh-negative when they are given blood transfusions, or in obstetrical problems related to the Rh factor. The use of small "r's" in designating these four types serves as a visual aid for remembering this fact [1]). As donors for Rh-negative patients, on the other hand, only individuals of type rh should be used, because type rh′ or type rh″ blood causes reactions in individuals sensitized to these blood factors.

Of the designation of the Rh blood types, the only ones that may cause even the slightest confusion are those for types Rh_1 and Rh_2. Type Rh_1 (or Rh'_0) is the designation of blood reacting with anti-rh′ and anti-Rh_0, but not anti-rh″ serum; while type Rh_2 (or Rh''_0) is the designation of blood reacting with anti-rh″ and anti-Rh_0, but not anti-rh′. The designations Rh_1 and Rh_2 were selected to indicate that the factors Rh_0 and rh′ in the former, and Rh_0 and rh″ in the latter, are usually combined to form unit agglutinogens inherited by corresponding genes R^1 and R^2.

For example, in mating of a type Rh_1 individual with a type rh individual, if type Rh_1 were really Rh_0rh′ then half the children would be expected to belong to type Rh_0 and half to type rh′. Actually, in some families all the children are type Rh_1; while in most other families half are type Rh_1 and half type rh. This occurence is explained by assuming that type rh individuals all belong to genotype *rr*, and that the type Rh_1 individuals in the former families are of genotype R^1R^1, while in the second type of family the genotype is R^1r. Thus, Rh_1 is merely an abbreviated designation for Rh'_0 while Rh_2 is an abbreviation for Rh''_0. Since medical men are already accustomed to numerous abbreviations,

[1]) For oral use, it is not necessary to say „large R" or „small r", as the case may be, because the qualifying subscripts and superscripts act as sufficient identification.

it is not too much to ask them to learn two more in order to streamline the nomenclature of the Rh blood types.

For the intelligent application of the Rh types in problems of erythroblastosis, some knowledge of their heredity is essential. Considering first the property Rh_0 alone, with which the clinician will be most often concerned, LANDSTEINER and WIENER have shown that it is transmitted as a simple Mendelian dominant, by a pair of allelic genes, *Rh* and *rh* [1]). Therefore, Rh-negative individuals are always homozygous (genotype *rhrh*) while Rh-positive individuals may be homozygous (genotype *RhRh*) or heterozygous (genotype *Rhrh*). Obviously, if both parents are Rh-negative, all the children must be Rh-negative. If one parent is Rh-negative and the other Rh-positive, there are two possibilities: either all the children will be Rh-positive (when the Rh-positive parent belongs to genotype *RhRh*), or half of the children will be Rh-positive and half Rh-negative (when the Rh-positive parents belong to genotype *Rhrh*). When both parents are Rh-positive all the children will be Rh-positive, except when both parents are heterozygous (genotype *Rhrh*), in which case three-fourths of the children will be Rh-positive and one-fourth Rh-negative.

To explain the heredity of the eight Rh types, a much more

Table II.

Eight Rh types and their twenty-one genotypes.

Rh blood types	Approximate frequency in N.Y.C. (%)	Possible genotypes
rh	13.0	rr
rh′	1.5	$r'r'$ and $r'r$
rh″	0.5	$r''r''$ and $r''r$
rh′rh″	0.01	$r'r''$
Rh_0	2.5	$R^\circ R^\circ$ and $R^\circ r$
Rh_1	52.5	R^1R^1, R^1r', R^1r, R^1R°, and $r'R^\circ$
Rh_2	15.5	R^2R^2, R^2r'', R^2r, R^2R°, and $r''R^\circ$
Rh_1Rh_2	14.5	R^1R^2, R^1r'', and $r'R^2$

[1]) Gene *rh* is not identical with the gene *r* discussed in the previous paragraph and given in table II. Gene *rh* is a „collective" gene and includes the genes *r*, *r′* and *r″*, just as the Rh-negative type includes types *rh*, *rh′*, *rh″*, and *rh′rh″*. Similarly, gene *Rh* is a „collective" gene and includes the genes R^1 R^2, and R°.

complicated scheme must be invoked. According to the theory proposed by WIENER the Rh blood types are transmitted by a series of six allelic genes, designated as R^1, R^2, R^0, r', r'', and r respectively, according to the agglutinogens which they determine, so that 21 genotypes are possible instead of only 3 (see table II). Knowledge of the Rh types makes it possible at times to determine that an individual is homozygous for the Rh_0 factor, information of importance when determining the prognosis in cases of erythroblastosis. For example, individuals of type Rh_1Rh_2 are usually homozygous (genotype R^1R^2), so that when the husband of an Rh-negative woman belongs to this type, one may predict with reasonable certainty that all the children will be Rh-positive (half type Rh_1 and half type Rh_2).

THE Hr BLOOD FACTORS

LEVINE and JAVERT demonstrated that Rh-negative bloods contain a special agglutinogen, designated Hr by them because of its apparently reciprocal relation to Rh. RACE and TAYLOR independently discovered an agglutinogen which they called St, and showed that St was related to rh' as M is related to N. WIENER has proved that Hr and St are identical, so that the designation St has been abandoned. Since the Hr antigen first found is reciprocally related to rh', WIENER suggested that it be designated as hr'. As FISHER pointed out, theoretically three Hr factors are possible corresponding to the three Rh factors. Anti-hr'' has been found by MOURANT, and more recently anti-Hr_0 has been found by HABERMAN, HILL, EVERIST and DAVENPORT.

Sensitization to the Hr factors can cause clinical complications similar to those produced by Rh sensitization, namely, intragroup hemolytic transfusion reactions and erythroblastosis fetalis. However, the Hr factors are far less antigenic than the Rh factors, so that such cases are quite rare. For the same reason, Hr antisera are much more difficult to procure than Rh antisera, and to date attempts to immunize Hr-negative donors in order to produce such antisera have met with little or no success. Yet Hr antisera are necessary to resolve clinical problems caused by sensitization to factors other than Rh, and for the selection of Hr-negative donors to be used for transfusing patients sensitized to one of the Hr factors. Another important application of the Hr antisera is for a

presumptive test for heterozygosity or homozygosity of Rh-positive husbands of Rh-negative prospective mothers, who are known to be sensitized to the Rh factor. Still another application is in disputed paternity cases, since the Rh-Hr tests considerably increase the chances of excluding a falsely accused man.

After a blood sample has been classified into one of the eight Rh blood types with the aid of the three Rh antisera, anti-rh′, anti-rh″, and anti-Rh_0, if indicated, the blood can be subtyped with the aid of anti-Hr sera. The Hr tests are done after the Rh tests in order to avoid unnecessary Hr testing, so as to conserve the rare and valuable Hr antisera. Thus, only bloods of types Rh_1 and rh′ need be tested with anti-hr′ serum, and, similarly, only bloods of types Rh_2 and rh″ need be tested with anti-hr″ serum. Other combinations uniformly give positive reactions, as shown in table III, barring bloods from the rare individuals who carry genes r^y and R^z. The Rh-Hr subtypes are designated as follows:

	Rh_1hr'	—	is called Rh_1Rh_1
	Rh_1hr'	+	is called Rh_1rh
	rh′hr′	—	is called rh′rh′
	rh′hr′	+	is called rh′rh
Similarly,	Rh_2hr''	—	is called Rh_2Rh_2
	Rh_2hr''	+	is called Rh_2rh
	rh″hr″	—	is called rh″rh″
and,	rh″hr″	+	is called rh″rh

It must be emphasized that these designations refer to phenotypes not genotypes. Thus, corresponding to phenotype Rh_1Rh_1, two genotypes, R^1R^1 and R^1r' are theoretically possible, while corresponding to phenotype Rh_1rh three genotypes, R^1r, $R'R^0$ and R^0r', are possible. Of these five genotypes, R^1R^1 and R^1r are by far the most common, and it will be seen that this is the basis for the selection of the phenotype names, namely, to indicate the most likely genotype. It is in this sense that anti-hr′ serum can be used to determine the probable genotype of type Rh_1 individuals, and anti-hr″ serum to determine the probable genotype of type Rh_2 individuals.

Both Hr antisera are available only in very limited amounts. Anti-hr′ is quite rare, while anti-hr″ is even rarer. To conserve these antisera further, therefore, it is recommended that the tests be done in very narrow tubes (inside diameter 4 to 5 mm instead of 7 to 8 mm). The additional difficulty caused by the use of such

Table III.

The Rh-Hr blood types and their corresponding genotypes.

Rh blood types [1]	Reaction with serum		Rh subtypes [1]	Approximate Frequency in N.Y.C. (%)	Possible genotypes
	Anti-hr′	Anti-hr″			
rh	*	*	rh	13.0	rr
rh′	—	*	rh′rh′	.01	$r'r'$
	+	*	rh′rh	1.0	$r'r$
rh″	*	—	rh″rh″	.005	$r''r''$
	*	+	rh″rh	0.5	$r''r$
rh′rh″	*	*	rh′rh″	.01	$r'r''$
Rh_0	*	*	Rh_0	2.0	R^0R^0 and R^0r
Rh_1	—	*	Rh_1Rh_1	20.0	R^1R^1 and R^1r'
	+	*	Rh_1rh	34.0	R^1r, R^1R^0 and R^0r'
Rh_2	*	—	Rh_2Rh_2	3.0	R^2R^2 and R^2r''
	*	+	Rh_2rh	12.0	R^2r, R^2R^0 and R^0r''
Rh_1Rh_2	*	*	Rh_1Rh_2	14.5	R^1R^2, R^1r'' and $r'R^2$

*) The asteriks indicate tests which need not be made, because the reactions in these combinations are invariably positive, except for types rh′rh″ and Rh_1Rh_2 in the case of individuals carrying the rare genes r^y and R^z. Should these tests be done and negative reactions obtained, then there has been an error in technique, and the entire examination should be repeated.

[1]) These are phenotypes and the symbols have been selected so as to indicate what tests have actually been done and what reactions were obtained. For example, ,,type Rh_1'' indicates that tests have been made only with sera anti-rh′, anti-rh″ and anti-Rh_0 and positive reactions obtained with anti-rh′ and anti-Rh_0, and a negative reaction with anti-rh″, on the other hand ,,type Rh_1Rh_1'' indicates type Rh_1 blood which has also been tested with anti-hr′ serum with which it gives a negative reaction.

narrow tubes is justified because of the scarcity of the antisera. In the case of the more plentiful Rh antisera the use of the wider tubes is advised because the latter are easier to manage.

NOMENCLATURE OF THE Rh-Hr TYPES

If the reader has carefully read the foregoing description of the Rh-Hr types, he will be prepared for the following analysis of the

Rh nomenclature "problem". It will become apparent that this "problem" has only been created artificially by those workers who have tried to introduce unnecessary and confusing duplicate and triplicate systems of notations. No problem exists for the worker who avoids the use of any but the original notations for the Rh-Hr types.

Advantages of the Rh-Hr nomenclature.

1. They have priority, having been proposed by one of the discoverers.
2. They form a coherent and unified system, which readily lends itself to extension to include newly discovered blood factors belonging to the rhesus system.
3. The genetic theory on which the notations are based is amply supported by heredity studies on a large series of families, and statistical studies on the distribution of the types in the population. On the other hand there is no evidence against the theory.
4. The notations are simple to use in writing and orally.
5. The notations clearly separate the designations for phenotypes and genotypes, and for agglutinogens and genes, leaving no room for ambiguity.
6. The phenotypes names clearly indicate what tests have actually been made and what reactions were obtained, without including symbols, for hypothetical blood factors which have not been tested for, and for which there is no clear experimental evidence.
7. The notations clearly indicate the relationship to the original rhesus factor, and separate the Rh-Hr types from other independent systems such as the A–B–O-groups and M–N types.
8. For individuals who already know and understand the four blood groups and the three M–N types, the Rh-Hr notations are easy to learn almost instantaneously.
9. The notations lend themselves readily to clinical use, since the capital R's and small r's clearly indicate which types are Rh_0-positive and which are Rh_0-negative.
10. They take into account not only the reciprocal relationship between the Rh and Hr factors, but also the special clinical, serologic and genetic position of factor Rh_0.
11. They do not involve any incorrect assumption of one-to-one correspondence between genes and partial antigens.

12. They are self-sufficient.
13. None of the symbols used in the Rh-Hr notations have previously been applied to other agglutinogens.
14. The notations as used by workers throughout the world are uniform.
15. Simple tables are available for the Rh-Hr types and their genetics.
16. They are the only notations used in most standard text books, encyclopedias, dictionaries and articles.

Supposed disadvantages of the Rh-Hr notations.

1. That they take longer to teach. Where this seems to be so, it is merely because one must understand the Rh-Hr types in order to use the Rh-Hr notations intelligently, and to teach them.
2. That they have been changed several times. This is true, but none of the changes are basic or really upsetting. Moreover, one need only learn the present nomenclature to understand the subject. The changes have served to improve and streamline the nomenclature, and are no more objectionable than the changes made from year to year in order to improve the performance and appearance of automobiles. One does not have to study the entire evolution of the automobile from 1905 to date in order to learn how to drive one's 1948 model.
3. That the notations of the genes and agglutinogens do not include the Hr factors. This is true, but for an excellent reason. As shown in table IV, each Hr factor is reciprocally related to the corresponding Rh factor, so that if the Rh factors determined by a gene are known, the Hr factors it determines are also apparent on inspection. For example, since gene R^1 determines the presence of factors Rh_0 and rh′ and the absence of rh″, it at the same time determines the absence of factors Hr_0 and hr′ and the presence of hr″. The situation is quite analogous to the reciprocal relation between agglutinogens and agglutinins in the LANDSTEINER blood groups. Yet the simple designations, O, A, B, and AB for the four blood groups have proved eminently satisfactory, and none but the neophyte feels the need for cumbersome designations such as O, anti-A, anti-B (or O$\alpha\beta$), or, A, anti-B (Aβ), etc.

Table IV.

Reactions determined by the Rh genes, including genes r^y and R^z.

Gene	Reactions with anti-Rh sera			Reactions with anti-Hr sera		
	Anti-rh′	Anti-rh″	Anti-Rh_0	Anti-hr′	Anti-hr″	Anti-Hr_0
r	—	—	—	+	+	+
r′	+	—	—	—	+	+
r″	—	+	—	+	—	+
r^y	+	+	—	—	—	+
R^0	—	—	+	+	+	—
R^1	+	—	+	—	+	—
R^2	—	+	+	+	—	—
R^z	+	+	+	—	—	—

Supposed advantages of the C-D-E- and Rh_1-Rh_2-Rh_3 notations.

1. Many workers have the false impression that they are easier to "learn" and teach. The reason for this is that one can speak about C-D-E and Rh_1-Rh_2-Rh_3 at once, without having any real understanding of the subject. These notations therefore serve as a convenient cloak for lack of knowledge and understanding, and actually discourage any desire to acquire such an understanding.
2. They make use of a one-to-one correspondence between genes and blood factors. This supposed advantage is based on the wrong concept that such a one-to-one correspondence must exist, which is not true.
3. They are gaining more adherents. This is true but only because the protagonists of this system are teaching it, and because the new regulation passed by the National Institute of Health has f o r c e a b l y called these notations to the attention of workers. The C-D-E and Rh_1-Rh_2-Rh_3 notations will naturally attract some adherents, just as the Moss and Jansky systems each gained their adherents. The resulting unnecessary confusion may take many years to clear.

Disadvantages of the C-D-E, Rh_1-Rh_2-Rh_3, and other similar systems.

1. They lack priority.
2. The genetic theory on which they are based is purely ethereal;

there is no experimental evidence to support it, nor does it lend itself readily to experimental attack. On the other hand, there is some statistical evidence based on the analysis of the distribution of the Rh-Hr types in the population that tends to refute the linkage idea.

3. The notations are cumbersome to use. For example, type rh becomes small-c, small-d, small-e, over small-c, small-d, small-e. An error in printing in which a capital letter is substituted for a small letter or vice versa could prove fatal. Thus if anti-d is written instead of anti-D this would be very serious, but if anti-rh_0 is written instead of anti-Rh_0 the intention of the writer would still be obvious.
4. There is no clear separation of phenotypes and genotypes. The use of designation for "the most likely" genotype without indicating clearly all the time what is intended conveys to the reader the false impression that the actual genotype has been determined. The writings of users of these notations continually confuse genotypes with phenotypes, and it is impossible to tell from the designation used what tests have actually been made.
5. The letters C-D-E give no indication of any relationship to the rhesus system and convey instead the erroneous impression of a relationship to the A-B system of blood groups.
6. To learn the other notations suggested one must start from beginning, and all one's knowledge and understanding of the A-B-O and M-N systems is wasted.
7. The notations are too awkward for clinical use. There is no clear indication in the notations which bloods are Rh_0-positive and which are Rh_0-negative.
8. While the C-D-E system of notations takes advantage of the reciprocal relationship between the Rh and Hr factors, it does not take into account the special clinical, serologic and genetic position of the factor Rh_0.
9. The notations are neither self-sufficient nor self-explanatory. Thus users of the C-D-E-notations are compelled also to use the Rh-Hr notations, as can be seen by consulting the papers of Fisher and Race who originated the C-D-E notations.
10. The symbols C and E have already been applied to other agglutinogens in human blood. The introduction of the use of these

letters for agglutinogens of the rhesus type would therefore result in ambiguity.

11. There is no uniform understanding among users of the C-D-E notations as to terminology for the phenotypes. Thus blood reacting with "anti-C", "anti-D" and "anti-c", but not with "anti-E" is variously designated as CcD, or CcDe, or CDe/cde, or (++—+), etc.

    Under the Rh-Hr notations, the blood would be designated simply as Rh_1rh, a name which clearly indicates positive reactions with sera anti-rh′, anti-Rh_0 and anti-hr′, and negative reactions with anti-rh″.

12. No simple unified tables regarding the types and their heredity involving the other proposed systems of notations are available for use.

13. The C-D-E and other proposed notations are not mentioned in most standard laboratory texts, and are therefore unintelligible to the average reader.

(Medical Research Council, Blood Group Research Unit, Lister Institute, London, S.W. 1.).

Rh BLOOD GROUPS: RESPONSE TO AN ATTACK

by

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At the end of 1943, WIENER (13) had isolated 6 allelomorphs of the Rh gene and defined their reactions with three different Rh antibodies; eighteen interactions in all.

At that time we had isolated 7 allelomorphs and defined 26 of their interactions with four different Rh antibodies (9,10), including all 18 worked out by WIENER. There was agreement where the two schemes overlapped. The extra antibody which we had employed, the anti-Hr of LEVINE or anti-St of RACE and TAYLOR (now called anti-c), was vital to the interpretation of the table of interactions. The table of interactions was bewildering; some allelomorphs reacted with the first serum, some with the second and third, some with the second and fourth, etc.

LEVINE (6) who had worked with the anti-Hr serum, had observed that no bloods were found which gave negative reactions with both anti-Rh_1 (as it was then called, now called anti-C) and the anti-Hr serum. This was indeed a step in the right direction, but was not published until 1945. An explanation of this delay is given in the paper referred to.

Studying the complex table of interactions of ,,allelomorphs'' and antibodies drawn up by the English workers (10), FISHER (3) noticed that those genes negative with the vital anti-St (Hr) serum (not included in WIENER's work) were all positive with the serum now called anti-C. FISHER said that the antigens recognised

[1]) I am grateful to the Editor of the Revue d'Hématologie for permission to publish this modification of a letter printed in that journal. The letter was a response to one of the many attacks made by Dr A. S. WIENER on the Rh work done in Britain.

by these two antibodies were allelomorphic and called them c and C.

The antigens recognised by the other two sera were not showing antithetical reactions. Consequently they were not allelomorphic antigens, so FISHER called them D and E. Like C they presumably had allelomorphs, d and e. FISHER then predicted that antisera anti-d and anti-e would be found corresponding to the antigens (or factors) d and e.

Following this, WIENER and SONN (16) wrote (May 18th, 1945): „FISHER has postulated the existence of two additional antisera and corresponding factors, which are related to Rh″ and Rh_0 respectively, in the same way that Hr is related to Rh′. On the other hand, WIENER suggests that Hr holds an unique position in the scheme of the Rh factors, analogous to the position of factor O in the scheme of the A–B–O agglutinogens (cf. WIENER and KAROWE), a view which would preclude the existence of the two additional factors postulated by FISHER. To date, no convincing evidence has been reported of the existence of antisera or factors corresponding to FISHER's hypothetical factors."

Subsequently FISHER's brilliant prediction was confirmed by the determination of two predicted reactions of the gene Rh_z (8) and, more dramatically, by the finding by MOURANT (7), of the anti-e serum, giving precisely the reactions predicted of it. This established the existence of the factor e in a positive way. More recently DIAMOND (2) and HILL and HABERMAN (5), have found the remaining antibody anti-d, and VAN DEN BOSCH (1) has discovered the predicted chromosome CdE.

WIENER thereafter adopted the three pairs of allelomorphic antigens (14), but without ceasing to attack the English work and FISHER's ideas (15). As FISHER's ideas have spread the more widely in the States, the violence of WIENER's attacks has increased, and the argument has been shifted from the essential allelomorphisms of the antigens (15, 17), which FISHER's theory postulated (which has been accepted by WIENER), to the precise structure of the gene or genes. On the latter subject the view to which FISHER inclines is the only one so far proposed with any evidence to support it. WIENER has many times stated that FISHER's theory has been proved to be wrong. It must be supposed that he is referring to the suggestion that the exact genetical structure may be three closely linked genes. To a balanced view it must be clear that it would be difficult to prove this suggestion right,

but almost impossible to prove it wrong. WIENER is attempting to disprove it by frequently repeating that it is disproved.

The following are the two notations for the "allelomorphs". FISHER's notation shews the three antigens which are produced by one Rh chromosome. WIENER's does not.

FISHER	WIENER	Criticism
CDe	Rh_1 or Rh_0 ← = C, ← = D	e ignored
cDE	Rh_2 or Rh_0 ← = E, ← = D	c ignored
cDe	Rh_0 ← = D	c & e ignored
cde	rh ← = ? d	c & e ignored
Cde	rh′ ← = C, ← = ? d	e ignored
cdE	rh″ ← = E, ← = ? d	c ignored

Other allelomorphs of D and C have since been found. A notation which is not supposed to be shorthand, should be able to take account of the real state of affairs. WIENER's notation ignores c, d and e which exist as certainly as do C, D and E. The notation is deficient even for the present state of certain knowledge. It has been stretched to cover the new allelomorph C^w, C^wDe being represented by Rh_0'''. But this cannot be further stretched to cover the two new allelomorphs, c^v and C^u (11,12). C^u would have to be, in C^uDe, Rh_0'''''' (still ignoring e).

C, c, C^w, c^v and C^u are allelomorphic antigens; a chrosomone can carry the gene for any one of them.

E and e are allelomorphic antigens; a chromosome can carry the gene for the one or the other.

C and E have not this fundamental mutually exclusive relationship; they are of another order of difference, for a chromosome can

carry both these genes. Therefore WIENER's ′, ″ and ′″ representing FISHER's C, E and C^w are confounding the orderly facts; are going against nature. The notation which will be useful for the next few years will be one that can cope with t h r e e sets of numerous a l t e r n a t i v e s, *e.g.* to avoid FISHER's notation,

$$\left.\begin{array}{l} a^1 \text{ or } a^2 \text{ or } a^3 \text{ or } a^4 \text{ or } a^5 \\ b^1 \text{ or } b^2 \text{ or } b^3 \text{ or } b^4 \text{ or } b^5 \\ c^1 \text{ or } c^2 \text{ or } c^3 \text{ or } c^4 \text{ or } c^5 \end{array}\right\} \text{ perhaps more}$$

FISHER's does it thus:

C c C^w c^v C^u
D d D^u
E e.

The disadvantage of such a notation as C^1 C^2 C^3 C^4 C^5, D^1 D^2 D^3 etc., etc., is that over 97 % of genotypes would receive the extra numerals unnecessarily.

FISHER's recognition of the allelomorphisms of the antigens was a brilliant advance which brought order into the chaos of the Rh antigen antibody reactions. It was based on work which had gone further in England than in the States. The use in the United States, until recently, of Dr. WIENER's notation alone, must there have been a serious handicap to the spread of knowledge. That there is now a strong reaction is indicated by the report of the Advisory Review Board on the Nomenclature of Anti-Rh Typing Serums (Science **107**, 27, 1948) and by the editorial in the American Journal of Pathology by HILL (4), the distinguished Rh worker and First President of the International Society of Hematology: "The adoption of this nomenclature and method of approach formulated as the result of the brilliant cooperative research of British workers in no way reflects on the earlier original discoveries of American investigators, but again emphasizes that science is international. . . . . In practical application with a large transfusion service, in teaching medical students and technicians, and even in explaining incompatibility problems of erythroblastosis or of transfusions to patients, we have found the CDE system to be most satisfactory."

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(From the Department of Bacteriology and Experimental Pathology of the Institute for Preventive Medicine, Leiden).

FAILURE TO CULTIVATE RABIES VIRUS IN VITRO

by

J. D. VERLINDE and A. KRET

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It has been shown by various workers that rabies virus can be cultivated in tissue cultures containing rabbit-, mouse-, rat- or chick-embryo brain in serum-Tyrode solution. Recently VEERARAGHAVAN (1, 2, 3) claimed to have grown rabies virus in a cell-free medium containing steamed sheep-brain extract, sheep serum, glycine and peptone. Addition of one or more accessory factors as tryptophan, thiamine hydrochloride, nicotinic acid, pyridoxine hydrochloride, calcium pantothenate, riboflavin and biotin, showed a stimulating effect on the growth of the virus. When all the accessory factors are added, the use of sheep serum and even of steamed sheep-brain extract in the medium could be dispensed with. Comparative tests carried out under aerobic and anaerobic conditions showed that the concentration of virus obtained in cultures incubated under strictly anaerobic conditions was higher than under aerobic conditions. If these findings have been interpreted correctly, they are of fundamental importance, and VEERARAGHAVAN (3) suggests that the etiological agent of rabies is not a virus in the accepted sense of the term.

Using VEERARAGHAVAN's slightly modified medium A (2) we have tried to confirm these findings.

Instead of emulsifying normal sheep's brain with a Waring blendor, it has been grinded in a mortar with glass powder. Though the virus content would be twice as high when brain extract and virus inoculum had been prepared with a Waring blendor, still 2.5 million m.l.d. will be left when either substance has been prepared by grinding. Sheep serum has been omitted, as VEERARAGHAVAN (1) obtained good results in a medium without serum. Our medium thus consisted of:

steamed sheep-brain extract, 10 per cent	7.5 ml
glycine, 20 per cent	6 ml
peptone, 1.5 per cent	5 ml
tryptophan, 2 mg per ml	0.5 ml
aneurine, 1 mg per ml	0.1 ml
nicotinic acid amide, 1 mg per ml	0.1 ml
pyridoxine hydrochloride, 1 mg per ml	0.1 ml
sodium pantothenate, 1 mg per ml	0.1 ml
lactoflavin, 1 mg per ml	0.1 ml
distilled water	24 ml

Instead of supplying VEERARAGHAVAN's virus inoculum, this being a 20 per cent suspension of the brain of a sheep completely paralysed after subdural inoculation with the Paris strain of rabies fixed virus, we used the virus inoculum prepared as follows:

The brain of a rabbit infected with the PASTEUR strain of rabies fixed virus was kindly supplied by Dr P. LÉPINE, Institut Pasteur, Paris, and passed by us through mice by intracerebral inoculation. The mice died regularly after 7 to 8 days. The supernatant from a 20 per cent suspension of the brains of mice completely paralysed after intracerebral inoculation of mouse passage virus was used for the inoculation of the culture medium.

The virus content of the culture was determined immediately

TABLE I.

	end dilution of virus						
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
virus control (mouse brain suspension in saline)	3/3	3/3	3/3	2/3	0/3	0/3	0/3
culture immediately after adding the virus inoculum	3/3	3/3	3/3	0/3	0/3	0/3	0/3
culture after 24 hours incubation	0/3	0/3	0/3	0/3	0/3	0/3	0/3
culture after 48 hours incubation	0/3	0/3	0/3	0/3	0/3	0/3	0/3

The denominator indicates the number of mice inoculated, the nominator indicates the number of mice which died.

after adding the virus, and after 24 and 48 hours incubation at 37° C. under aerobic conditions, by inoculating mice intracerebrally with 0.02 ml of various dilutions of the medium in saline. The medium was diluted in a measure that the end dilutions of virus varied from 10^{-2} to 10^{-8}. The results of the virus titrations are recorded in table 1.

They reveal that we have not been able to confirm VEERARAGHAVAN's results. No virus multiplication could be shown, on the contrary the virus lost its virulence completely within 24 hours.

The mice that survived the inoculation with the 24 and the 48 hours culture have been reinoculated intracerebrally with living virus in order to check immunity. All these animals, however, died after 8 to 10 days, indicating that no immunity had developed.

Summary.

Report of the unsuccessful cultivation of rabies fixed virus in a cell-free medium consisting of steamed sheep-brain extract, glycine, peptone and a number of accessory factors (tryptophan, aneurine, nicotinic acid amide, pyridoxine hydrochloride, sodium pantothenate and lactoflavin). After 24 and 48 hour's incubation at 37° C, under aerobic conditions, the virus had completely lost its virulence, and did not induce immunity to reinoculation with active virus.

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(From the „Rijks Instituut voor de Volksgezondheid" at Utrecht).

STUDIES ON STAPHYLOCOCCI

III. STAPHYLOCOAGULASE, AN ANTIGEN?

by

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(Received December 22, 1948).

The ability to coagulate plasma *in vitro* is generally considered an important characteristic of the pathogenic staphylococci (1); also the importance of staphylocoagulase has been demonstrated for the pathogenicity *in vivo*, *viz.*, for the initiation and development of infections by this organism (HALE and SMITH (7), SMITH, HALE and SMITH (11)).

No unanimity has been attained as yet in the answering of the question whether staphylocoagulase possesses antigenic properties and whether in the blood specific antibodies neutralizing this substance might occur.

In a previous communication (2), in accordance with the observations of GROSS (6) and of LOMINSKI and ROBERTS (9) it was stated that in human serum a substance can be found capable of inhibiting the clotting of plasma by staphylocoagulase *in vitro*. This observation has recently been confirmed by BRUINING and COHEN (3).

This antistaphylocoagulase, not to be confounded with the anticoagulase of CHAPMAN, BERENS and STILES (4) showed antibody characteristics, it was however probably not linked with the γ-globulin fraction of the serum, which contains most of the antibodies, but with the α- or β-fraction.

WALSTON (14) and SMITH and HALE (10) did not succeed in immunizing their test animals by means of staphylocoagulase.

The description of new methods for the preparation, the purification and the concentration of staphylocoagulase (GERHEIM,

Ferguson and Travis (5) and Walker, Derow and Schaffer (13)) made me resume the attempt to induce the formation of antistaphylocoagulase.

Technique. Staphylocoagulase was prepared according to the method of Lominski (8) by cultivation of a strain of *Staphylococcus aureus* during 24 hours at 37° C. in rabbitplasma-broth, followed by a filtration through an L 3 candle; and according to the method of Gerheim (5) by precipitation of an 48 hours culture of *Staphylococcus aureus* with 95 per cent ethylalcohol at 0° C.

The formation of antistaphylocoagulase was estimated as described previously (2) by mixing 0.5 ml serial doubling dilutions of serum (either heated for 30 min. at 56° C. or unheated) with 0.2 ml staphylocoagulase (20—30 M.C.D.); after incubating for 90 min. at 37° C. 0.3 ml rabbitplasma (1:3 in saline) were added; the mixture was incubated for 24 hours at 37° C. and the degree of clotting noted.

Results. For the immunization experiments rabbits were used in the serum of which previous to the experiment no antistaphylocoagulase had been traced. The method of inoculation, the nature and the quantity of staphylocoagulase inoculated and the interval between the inoculations varied in the different experimental series.

1st series: 10 rabbits received each 8 intravenous inoculations of 0.25 to 3 ml staphylocoagulase according to Lominski; an inoculation once a week.

2nd series: 10 rabbits received each 8 subcutaneous inoculations of 0.25 to 3 ml staphylocoagulase according to Lominski; an inoculation once a week.

3rd series: 10 rabbits received each 8 intravenous inoculations of 0.5 to 2 ml of a 1 % solution of staphylocoagulase according to Gerheim c.a. (in citrate-borate buffer); an inoculation once a week.

4th series: 5 rabbits from the 1st series and 5 from the 2nd series received each 30 intramuscular inoculations of 5 ml staphylocoagulase according to Lominski; every 6th day.

5th series: 5 rabbits from the 1st series and 5 from the 2nd series received each 30 intramuscular inoculations of 5 ml staphylocoagulase according to Gerheim c.a. (1 % solution); every 6th day.

6th series: 5 rabbits from the 4th series received moreover 15

intraperitoneal inoculations of 5 ml staphylocoagulase according to GERHEIM c.a. (1 % solution); every 4th day.

7th series: 5 rabbits from the 5th series received moreover 15 intraperitoneal inoculations of 5 ml staphylocoagulase according to GERHEIM c.a. (1 % solution); every 4th day.

All these experiments offered negative results. In none of the rabbits treated any formation of antistaphylocoagulase could be demonstrated after the administration of staphylocoagulase.

Discussion. From the above mentioned experiments it may be concluded that staphylocoagulase as such does not act antiginetically, although it may still be considered a possibility, that staphylocoagulase might act as a hapten, it only inducing the formation of antistaphylocoagulase in the presence of a carrier protein.

The inhibitory action of some human sera on the plasmacoagulation by staphylocoagulase therefore may hardly be explained as resulting from an antigen-antibody reaction between staphylocoagulase and antistaphylocoagulase.

Recent investigations have made it probable that the clotting of plasma by staphylocoagulase requires an interaction of (pro)-staphylocoagulase formed by the bacteria and an activator substance (SMITH and HALE (10)), occurring in the plasma-albumins (GERHEIM c.a. (5)) and in the plasma-globulins (TAGER (12)).

According to SMITH, HALE and SMITH (11) the inhibition of the plasmacoagulation might be the result of some inhibition or destruction of the coagulase-activating substance. I could note (2) that after addition of fresh plasma unto a non-clotted mixture of serum, staphylocoagulase and plasma coagulation occurred. This phenomenon was explained as being a manifestation of the reversibility of the fixation of staphylocoagulase with antistaphylocoagulase, but might as well be explained by a neutralization of the inhibition of the activating substance by the addition of fresh activating substance.

An explanation of the inhibition of the plasma coagulation either by action on staphylocoagulase, by action on the coagulase-activating substance or by some other cause, will be only arrived at after a careful study of the nature of staphylocoagulase and of the activating substance.

Summary.

In immunization experiments no antigenicity of staphylocoagulase could be demonstrated. The action of antistaphylocoagulase is discussed.

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ACTIVATION OF INAPPARENT INFECTION WITH ECTROMELIA AND PNEUMONIA VIRUS IN MICE

by

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Intranasal instillations in mice and other experimental animals may give rise to a viruspneumonia which erroneously might be ascribed to the action of a virus in the original material.

In 1944 we found peculiar lunglesions in mice used for the mouse adaptation of influenza virus. The intranasal instillation of animals from a mouse colony with certain strains of influenza A virus recently isolated on ferrets, resulted in the death of some mice within 2 to 8 days. The lungs showed greyish foci which were quite different from the lesions in experimental influenza. Similar lesions, however, were found in apparently normal mice from the same colony, and a few weeks later, the undeniable symptoms of ectromelia appeared, a virus disease so far not described in Holland.

Some of the sick animals showed edematous swelling, ulcerations, necrosis and amputation of one or both hindlegs. In a few cases one or both forelegs, the tail and the snout were affected. At autopsy sharply outlined greyish lesions in the lungs and the liver, and swelling of the spleen were found. The alveolar walls were thickened and infiltrated with leucocytes, the alveoli were filled with leucocytes and desquamated epithelium, the bronchi in these foci contained leucocytes, but the bronchial wall appeared to be unaltered. Central necrosis was observed in many foci. In the liver a fairly large number of necrotic areas without evident cellular reaction was present. There was a considerable thickening of the

[1]) Communication of the Influenza Workteam of the Institute for Preventive Medicine.

epidermis, subcutaneous edema, leucocytic infiltration and necrosis of the affected legs.

Mice could readily be infected by intradermal inoculation on the metatarsal region with a drop of edema fluid or lungsuspension which contained the causative virus detected by MARCHAL (12). Three to four days after inoculation a local swelling appeared, followed by a rapidly developing affection, being clinically as well as histologically quite similar to the spontaneous disease. The experimentally infected mice died within 7 to 12 days.

In sections of the epidermis of animals suffering from the spontaneous disease as well as in the experimentally infected mice, stained by MANN's or by LENTZ's method, acidophylic, ellipsoid or spherical inclusion bodies of various size and without internal structure were found. Smears of the edema fluid and of the lungs, coloured by GIEMSA-, PASCHEN- or HERZBERG-stain, contained large accumulations of elementary bodies.

The intradermal inoculation of mice from the naturally infected colony proved unsuccessful. It is assumed that many animals of an infected colony pass through a subclinical infection from which they acquire an active immunity. A natural immunity does not occur.

According to KIKUTH and GÖNNERT (11) widespread inapparent infections would occur, and clinical manifestations would appear especially after intranasal instillation of influenza virus. Our observations may support this view.

In mice and other animals latent infections with other viruses may occur among which those of the psittacosis-lymphogranuloma group are most important. These too may provoke a viruspneumonia after intranasal instillation of arbitrary material. The viruses of this group are characterized by relatively large elementary bodies which are stained well by GIEMSA-, CASTANEDA-, and MACHIAVELLO-stain.

FRANCIS and MAGILL (7) isolated the meningopneumonitis virus from the lungs of ferrets after they had been inoculated intranasally with throat washings from patients suffering from an influenza-like disease. This virus caused a pneumonia in mice, but it lost its virulence after some animal passages. It has not been shown with certainty whether the meningopneumonitis virus was from human or from ferret origin. Moreover EATON, BECK and PEARSON (5), EATON, MEIKLEJOHN, VAN HERICK and TALBOT (6), and OLSON and

Larson (14) isolated three viruses by intranasal instillation of mice and cottonrats with sputum from patients suffering from primary atypical pneumonia. Strict evidence, however, that these viruses are of human origin, has not been furnished.

Viruspneumonitis in mice and cats has to be classed in this group too. Viruspneumonia in mice has been observed in the Netherlands by de Blieck (3) and in other countries a.o. by Dochez, Mills and Mulliken (4), Gordon, Freemann and Clampit (9) and by Gönnert (8). Baker (2) described viruspneumonia in cats.

The pneumonia virus of mice is of particular interest, as the mouse is the usual experimental animal in pneumotropic virus research. It hardly needs recalling that adaptation of influenza virus and the mouseprotection test in influenza immunology are frequently carried out. Further the resuscitation of the experimental rickettsia-pneumonia by intranasal infection of mice with typhus and other rickettsiae is the classical method for obtaining a rich culture of these organisms, and for preparing rickettsia-vaccines. It has been shown in experiments set up for the isolation of the causative agent of primary atypical pneumonia and of other respiratory diseases in man, that mice can be carriers of the mouse pneumonia virus, and that this virus can be activated by intranasal instillation, from which a viruspneumonia may result. At autopsy and even after histological examination, the differentiation of virus and rickettsial pneumonias may be very difficult (Mooser (13), Aufdermauer (1)), perhaps with the exception of experimental influenza pneumonia (Straub (15)). Extensive control experiments intending to exclude latent infections with the pneumonia virus are therefore necessary before drawing far-reaching conclusions with regard to the origin of the isolated virus. By describing a series of experiments we want to substantiate this view.

In a small rural municipality a fairly large number of cases of acute respiratory disease occurred in children as well as in adults. The initial surmise of primary atypical pneumonia was abandoned on account of the clinical picture and the constantly negative cold agglutination.

We failed to isolate influenza virus from the throat washings in the acute stage by the usual procedures. At the same time fresh defibrinated blood and throat washings, obtained by gargling with

broth-saline, were examined as to the presence of a virus from the psittacosis-lymphogranuloma group. The blood proved to be bacteriologically sterile, the throat washings were treated with 250 units penicillin and 2.5 mg streptomycin per ml.

Six groups of 8 mice were inoculated intracerebrally, intranasally and intraperitoneally either with blood or with throat washings from three patients on the first day of illness. As none of the animals showed any reaction within 10 days, 4 out of each group were killed. The brains, the spleen and the lungs were removed aseptically, and suspensions of them were inoculated intracerebrally, intranasally and intraperitoneally into three groups of 8 fresh mice. In smears of the organs coloured with GIEMSA- and MACHIAVELLO-stain no elementary bodies were observed. Three subsequent intracerebral and intraperitoneal passages were entirely negative. Thus the presence of psittacosis or ornithosis virus appeared highly unlikely.

The intranasal instillation of throat washings and subsequent blind passages with mouse lung suspensions resulted in the death of one out of 3 mice from the third passage, 3 days after instillation. The lungs showed edema and a velvety, blue-reddish coloured surface. In GIEMSA- and MACHIAVELLO-stained smears of the lungs elementary body-like granules were found.

We readily succeeded in making serial lung passages by intranasal instillation. All mice died within 3 days, and elementary bodies could be demonstrated regularly in stained smears of the lungs.

In one group of 8 mice, 2 died 7 days after intranasal instillation of patient's blood. The lungs showed a same picture as those of the mice in the throat washings series, though less extensively. In stained smears no elementary bodies were demonstrated. In 6 subsequent passages most of the mice died after a gradually shortening incubation period. From the 7th passage all mice died within 3 days after infection. Elementary bodies could be detected in the lungs from the 4th passage onward.

In the same period mouse passages were made with influenza B virus with the view to adapt this strain onto mice. Three out of 6 mice in each passage were killed 2 to 4 days after the intranasal instillation; the others as far as surviving were killed after 10 days.

In the first passage no lunglesions were present either in the 3 mice killed after 2 to 4 days, or in those killed after 10 days. In

the second passage the 3 mice which had not been killed after 2 to 4 days, died after 6, 7 and 7 days, and in the third passage after 3, 6 and 7 days respectively. The lungs of all animals showed typical influenza lesions. In the 4th passage one mouse died as early as 1 day after the intranasal instillation, and lunglesions resembling those in experimental influenza were found. As a matter of fact, mice never die from influenza-pneumonia within 2 days, however high the virulence may be. In accordance with the experience gathered experimentally an accidental infection was surmised. The lungs proved bacteriologically sterile, but in GIEMSA- and MACHIAVELLO-stained smears elementary bodies were demonstrated. This agent was readily transmissible in serial passages.

After one or more passages the agent caused death of the mice in all series within 3 days. Signs of illness appeared within 48 hours: the animals refused food, showed bristling hair, a forced respiration and strong emaciation. The lungs showed blue-reddish edematous foci, usually beginning in the anterior lobes and extending backwards. The histological examination revealed consolidation and areas in which the alveoli were filled with edema fluid and leucocytes. The alveolar walls were thickened by cellular infiltration. The bronchi contained exudate, yet the bronchial wall appeared unaltered.

Though the serial passages from the throat washings and the blood of the patients and those of the influenza B virus pointed to the activation of mouse pneumonia virus, we still made three different series of intranasal transmission with normal mouse lungsuspension. In the first series pneumonia and elementary bodies appeared as early as the second passage, in the second series after the 6th passage, whereas the 7 passages of the third series were entirely negative. In this way we detected 7 healthy carriers among 67 apparently normal mice, *i.e.*, 10.4 per cent.

Though the mouse pneumonia virus presumably did not have any connection with the epidemic of acute respiratory disease in man, we examined acute phase serum of 3 patients, 3 convalescent sera and serum of 3 normal persons from a different region as to the presence of neutralizing antibodies against this virus, this in consequence of a communication by HORSFALL, CURNEN, MERICK, THOMAS and ZIEGLER (10) who demonstrated antibodies against mouse pneumonia virus in convalescent serum of persons recovered from primary atypical pneumonia.

Tenfold dilutions of virulent mouse lung suspension were mixed with equal parts of undiluted serum. The mixtures were placed one hour at room temperature, and then 4 mice were infected intranasally with each of them. The surviving animals were killed 8 days after the instillation. The measure in which lunglesions occurred is recorded in table 1

TABLE I.

Serum	mice	end dilution of virus				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Horse serum 10% (control)	1	++++	++++	++++	++	—
	2	++++	++++	++++	++	—
	3	++++	++++	++++	+	—
	4	++++	++++	++++	+	—
acute phase serum 1	1	++++	++++	++	—	—
	2	++++	++++	+	—	—
	3	++++	+++	—	—	—
	4	++++	+++	—	—	—
acute phase serum 2	1	++++	++	—	—	—
	2	++++	++	—	—	—
	3	++++	+	—	—	—
	4	++++	+	—	—	—
acute phase serum 3	1	++++	++++	+	—	—
	2	++++	+++	—	—	—
	3	++++	++	—	—	—
	4	++++	++	—	—	—
convalescent serum 4	1	++++	++++	++	—	—
	2	++++	++++	+++	—	—
	3	++++	++++	++	—	—
	4	++++	+++	+	—	—
convalescent serum 5	1	+++	++++	+	—	—
	2	+++	+++	+	—	—
	3	+++	+++	+	—	—
	4	+++	+++	+	—	—
convalescent serum 6	1	++++	++++	++	—	—
	2	+++	+++	+	—	—
	3	+++	+++	—	—	—
	4	+++	+++	+	—	—

TABLE I (continuation)

Serum	mice	end dilution of virus				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
normal serum 7	1	++	++	—	—	—
	2	++	+	—	—	—
	3	++	+	—	—	—
	4	++	+	—	—	—
normal serum 8	1	++++	+	—	—	—
	2	++++	+	—	—	—
	3	+++	+	—	—	—
	4	+++	—	—	—	—
normal serum 9	1	++++	++	—	—	—
	2	++	+	—	—	—
	3	+++	+	—	—	—
	4	+++	—	—	—	—

— no lung lesions; + lesions extending over 1/4 of the lungs; ++ lesions extending over ½ of the lungs; +++ lesions extending over 3/4 of the lungs; ++++ lesions extending over the entire lungs.

It appeared that the patient's as well as the normal sera had up to a certain extent a neutralizing activity against the mouse pneumonia virus. There was no significant difference between the neutralizing action of acute phase and convalescent serum, so that also this experiment leaves no doubt that the mouse pneumonia virus does not have any etiological relationship with the acute respiratory disease in man.

It seems likely that the intranasal instillation of patient's blood in mice being carriers has weakened the pathogenicity of the virus by the neutralizing power of the serum. This might explain the fact that the initial incubation period in the bloodpassage series was longer than in the other series.

Summary.

In mice used for the adaptation of a strain of influenza A virus, peculiar lunglesions, differing from influenza lesions, were found. Afterwards cases of ectromelia were observed in the same mouse colony, and the ectromelia virus was demonstrated in the clinically

manifest cases as well as in the lunglesions of apparently normal mice that died after intranasal instillation of influenza virus.

A few years later mice from a different colony were used for influenza B virus adaptation and in attempts to demonstrate a virus in the blood and throat washings of persons suffering from an acute respiratory disease, which prevailed in a rural municipality. In a number of mice that had been infected intranasally with influenza B virus, and with defibrinated blood and throat washings of patients, a pneumonia developed after some blind passages, which appeared to be caused by the mouse pneumonia virus. 10.4 per cent of apparently normal mice in the colony proved to be carriers of the virus. The causative agent of the epidemic in the village could not be detected. In convalescent as well as in acute phase and in normal sera from persons in a different region, neutralizing antibodies against the mouse pneumonia virus could be demonstrated.

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CELLULOSE-DECOMPOSING BACTERIA FROM THE RUMEN OF CATTLE[1])

by

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(Received April 16, 1949).

When in 1943, the present work was started there appeared to be a considerable discrepancy as to the nature of the bacteria that account for the cellulose breakdown in the rumen of ruminants and in the caecum and colon of other herbivorous mammals.

Experiments of KHOUVINE (8), POCHON (11), and several other workers had shown that various kinds of spore-forming, cellulose-decomposing bacteria were obtained after anaerobic incubation of cellulose-containing media, inoculated with contents of the organs in question.

These bacteria were of the same plectridial type as those accounting for the anaerobic breakdown of cellulose in soil and water. This result led to the idea that a rôle of importance in cellulose-breakdown in animals should be ascribed to them.

HENNEBERG (6), however, and more recently BAKER (various publications since 1931; see review of BAKER and HARRISS (2)) reported that microscopic examination of the contents of rumen and caecum of diverse herbivorous mammals revealed predominantly different kinds of cocci on cellulosic tissue fragments. BAKER also mentioned the occurence of slender vibrios.

VARTIOVAARA and ROINE (12) working with cellulose-decomposing bacteria from the caecum of pigs reported briefly that their cultures consisted mainly of two types of bacteria: small Gram-negative rods; and Gram-positive cocci occurring in pairs or chains.

During the course of the author's own investigations spore-forming cellulose-decomposing bacteria were commonly recovered from various media inoculated with the rumen contents of cattle.

1) Abstract of Thesis, Leiden 1948.

However, as ANKERSMIT (1) emphasized in 1906, these bacteria did not seem to occur in great numbers in the rumen as habitat. No cellulose decomposition could be obtained with inocula of less than 0.05 ml of rumen contents.

Further experiments showed that in media supplemented with a reducing agent and growth factors the cellulose was attacked by various kinds of cocci (Fig. 1—4) or occasionally by small slender, non-sporing rods but never by plectridia. Microscopic examination of rumen contents fully confirmed the reports of HENNEBERG and BAKER that cocci predominate on cellulosic materials.

It seems clear that the spore-forming type of cellulose bacteria do not play any important rôle in cellulose breakdown in the rumen. They should be considered as passengers ingested with the food, but unable to develop under the conditions prevailing in the rumen.

Dilution series in agar media, with strips of filter paper as a substrate, showed that over 6 million cellulose-decomposing cocci may be present per ml rumen contents.

Two types of cocci which occurred in great numbers were studied in detail. Neither could be included in any accepted genus. Moreover, their mutual differences were such that they could not belong to one genus. Therefore the introduction of two new genera, *Ruminobacter* and *Ruminococcus* seemed inevitable.

The name *Ruminobacter parvum* has been proposed for a small slightly oval, Gram-negative coccus, $0.5 \times 0.8\ \mu$ in size (Fig. 1). Unfortunately, it proved impossible to isolate them in pure culture. Under an atmosphere of hydrogen, highly purified cultures of two strains yielded propionic and acetic acids as the main products of cellulose decomposition. Though strict proof is lacking, there is reason to assume that these acids were direct end products of the activity of *Ruminobacter parvum* on the cellulose. Gas production was very restricted.

The position of the genus *Ruminobacter* has had to remain undecided for the time being.

A Gram-positive, catalase-negative streptococcus, for which the name *Ruminococcus flavefaciens* has been proposed was obtained in pure culture. A yellow pigment was formed by the culture. The diameter of the cells was $0.8—0.9\ \mu$ (Fig. 2 and 3). Unfortunately, growth in pure culture was very scanty, and for this reason no quantitative estimations of the end products of cellulose decompo-

sition could be made. However, in combined culture with a clostridium, identified as *Cl. sporogenes*, *Ruminococcus flavefaciens* rapidly broke down the cellulose. In one experiment carried out in an atmosphere of pure carbon dioxide, 4.15 m. mol. cellulose was decomposed in the presence of a mixed culture of *Ruminococcus flavefaciens* and *Cl. sporogenes*. The following end products were recovered: 0.94 m. mol. formic acid, 3.26 m. mol. acetic acid, 0.58 m. mol. butyric acid, 1.12 m. mol. lactic acid and 2.41 m. mol. succinic acid. Little or no gas was produced.

The genus *Ruminococcus* should undoubtedly be included in the tribe *Streptococceae* in the taxonomy proposed by KLUYVER and VAN NIEL (9).

In 1946 and 1947 important papers were published by HUNGATE (7) who reported the isolation of pure cultures of several strains of Gram-negative cellulose-decomposing cocci and small non-sporing rods from the rumen of cattle. Part of his excellent technique was used by the author in the later stages of her investigations. No mention, was made by HUNGATE of the fermentation products arising from his cultures.

Ruminobacter parvum might well be identical with any of the cocci described by HUNGATE; whereas *Ruminococcus flavefaciens* might be identical with the streptococci mentioned by VARTIOVAARA and ROINE.

The volatile acids i.e. acetic and propionic acids, accumulating in cultures of *Ruminobacter parvum* were the same as those found in the rumen by ELSDEN (3), ELSDEN *et al.* (4); see also ELSDEN and PHILLIPSON (5). These workers and also MARSTON (10), found that the same volatile acids were produced in enrichment cultures of cellulose bacteria from the rumen made under conditions closely resembling those prevailing *in vivo*.

In the case of *Ruminococcus flavefaciens* the situation seems to be less clear. Succinic acid, which is probably not present in rumen contents appeared as main product in highly purified cultures of this bacterium. However, the possibility is not excluded that any succinic acid produced in the rumen may be converted into propionic acid by the propionic acid producing bacteria, shown to be present in rumen contents.

The present investigations were carried out at the „Laboratorium voor Microbiologie" of the Technical University at Delft and were supported by the „Technische Afdeling" of the „Organisatie voor Toegepast Natuur-

wetenschappelijk Onderzoek''. The author wishes to thank Mr. J. A. Schuur for his able assistance in making the micrographs.

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HAEMAGGLUTINATION DURCH POCKENVIRUS

5. Die Erzeugung von Antistoffen gegen Vaccinevirus-Haemagglutinine bei Meerschweinchen und Ratten [1])

von

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(Eingegangen am 5 April 1949).

In einer früheren Mitteilung war über Versuche berichtet, die Haemagglutination durch Vaccinevirus mittels Kaninchenantisera zu hemmen. Die Kaninchen waren mit frischem Pockenvirus vom Karbouw intravenös an 6 aufeinanderfolgenden Tagen behandelt und lieferten in der Tat auch brauchbare Antisera, wenn die erzielten Titer in der Regel auch recht niedrig blieben.

Es schien interessant, vergleichsweise auch Antisera von anderen Tierspecies zu untersuchen. Ausserdem hatte sich gezeigt, dass in der Lunge verschiedener Tierarten Rezeptoren vorhanden waren, die eine hohe Affinität zum Vaccinevirus aufwiesen. Infolgedessen war es wünschenswert nachzugehen, ob durch pulmonale Immunisierung ebenfalls im Serum Antistoffe gegen die Haemagglutinine aufträten und ob diese besondere Eigenschaften besässen.

Technik.

Als Versuchstiere dienten Meerschweinchen von ca. 250—300 g und noch nicht völlig ausgewachsene schwarz-weisse Ratten von ca. 80 g. In zwei Versuchsserien wurden auch Ratten von ca. 50 g benutzt. Die Versuche wurden in der trockenen Zeit (± 22° C.) begonnen und in der Regenzeit (± 18° C.) beendet, doch zeigte sich hierbei keinerlei Unterschied im Ablauf der Versuche.

Zur Immunisierung wurde Vaccinevirus vom Karbouw benutzt, das drei Tage nach cutaner Impfung gewonnen war. Das Virus wurde ohne Glyzerinzusatz bei —15° C. aufbewahrt. Direct vor Gebrauch wurde das in der Chalybaeusmühle fein gemachte Material im Mörser mit physiologische Kochsalzlösung zu einer 10%igen

[1]) Mitteilungen 1, 2, 3 und 4: Documenta Tropica 1949 (in press).

Suspension verrieben und, ca. 10 Minuten lang bei 2000 Touren centrifugiert. Die überstehende Flüssigkeit wurde zur Immunisierung benutzt.

Die Immunisierung erfolgte subcutan oder intraperitoneal oder pulmonal in leichter Aethernarkose. Verschiedene Meerschweinchen wurden auch nur durch Augenskarifikation immunisiert.

Nach verschiedenen Intervallen wurden die Tiere entweder entblutet oder es wurde durch Herzpunktion Blut gewonnen. Das Serum wurde abzentrifugiert und bis zum Gebrauch bei —15° C. bewahrt.

Das Blut für die Agglutination stammte von javanischen Landhühnern, wurde in Citratauflösung aufgefangen und dreimal gewaschen. Meist war es frisch, nur gelegentlich 24 Stunden lang bei 4° C. bewahrt.

Zur Prüfung der Antistoffe wurde zuerst im Vorversuch die Virusverdünnung (Karbouwenpulpa) festgestellt, durch die gerade noch eine deutlich erkennbare Haemagglutination erzielt wurde. In der Regel wurde dann mit dem zweifachen und vierfachen Multiplum hiervon der Versuch angesetzt.

Von den zu untersuchenden Seren wurden 3 Serien fallender Verdünnungen hergestellt, beginnend mit 1/1,66, 1/3,33 usw. oder 1/16,6 1/33,3 usw. und zwar in der Menge von 0,2 ccm. Hierzu kam 0,2 ccm der Virusverdünnungen und zwar in der 1. Reihe das Vierfache und in der 2. Reihe das Doppelte der grade noch agglutinierenden Konzentration. Zur 3. Reihe kam zur Kontrolle von spontaner Serum-Haemagglutination 0,2 ccm physiologische Kochsalzlösung. Die Bindung von Serum und Virus erfolgte bei 37° C. 30 Minuten lang. Hierauf kam zu jedem Röhrchen 0,2 ccm 1,5 % Hühnerblut. Nach ca. 1½ Stunden bei Zimmertemperatur konnte abgelesen werden. Die Endverdünnungen des Serums betrugen stets 1/5, 1/10 usw. oder 1/50, 1/100 usw.

Versuche am Meerschweinchen.

Zu Beginn der Versuche wurde von der Annahme ausgegangen, dass eine wiederholte Immunisierung notwendig wäre, um höhere Antistofftiter zu erhalten. In Tabelle 1 finden sich daher Versuche mit zweimaliger Immunisierung aufgenommen. In allen drei Serien geht der Titer von 1/50 bis 1/2560.

In der gleichen Tabelle finden sich weiterhin Versuche über Immunisierung durch Augenskarifikation. Hier handelt es sich um

Tabelle 1.

Antistoffe gegen Vaccinevirus-Haemagglutination beim Meerschweinchen.

Immunisierung	Intervall in Tagen	Anzahl der Tiere mit Serumtiter von:											
		1/5=0	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	Total
2×0,2 1/10 sbc.	10–20					1	1	4		1		1	8
2×0,2 1/10 ip.	10–20						1	1	2	7	5	4	20
	21–40							1	2	1	1	2	7
2×0,5 1/10 ip.	10–20						1	4	4	1	1	2	13
	21–40							1	1	3	1	2	8
Augen-Skarifikation	21–40	7	3	4	8	8	4	5	3				42
	41–80	10	2	4	4		1						21
Negative Augenskarifikation	21–40	19	1										20
	41–80	21											21
Normale Caviae	—	8											8

Tiere, die zwecks Virustitration mit verschiedenen Vaccineverdünnungen behandelt worden waren und sehr deutliche Augenerscheinungen entwickelt hatten. Naturgemäss war die Viruskonzentration hier ganz erheblich schwächer als bei den subcutan oder intraperitoneal vorbehandelten Meerschweinchen (1/500 bis 1/50.000). Wenn daher 17 Tiere selbst in der Serumverdünnung von 1/5 keine Antistoffe aufwiesen, so ging bei einzelnen Tieren der Titer bis zu 1/320.

Weiterhin sind 41 Tiere besonders angeführt, bei denen nach Augenskarifikation keinerlei lokale Erscheinungen aufgetreten waren. Von diesen zeigten 40 in der Verdünnung 1/5 keine Antistoffe, und eines nur in der Verdünnung von 1/5.

Schliesslich findet sich noch eine kleine Kontrollserie von 8 normalen Meerschweinchen, von denen keines einen Titer von 1/5 aufweist.

In der nachfolgenden Tabelle 2 sind nunmehr die Versuche zusammengestellt, in denen von einer Serie Meerschweinchen ein Teil subcutan, ein Teil intraperitoneal und ein Teil pulmonal unter Aethernarkose jeweils mit 0,5 ccm 1/10 Virussuspension infiziert worden war. Nach verschiedenen Intervallen wurde das Serum dieser Tiere auf Antistoffe gegen Vaccinevirus-Haemagglutination untersucht. Es zeigte sich, dass nach 5 Tagen in keinem Fall Antistoffe nachgewiesen werden konnten. Nach 7 und 8 Tagen finden sich noch in allen drei Gruppen Tiere, die bei Serumverdünnung 1/5 keine Antistoffe haben, doch wird schon gelegentlich ein Maximum von 1/640 erreicht. Wird der Durchschnitt errechnet, wobei die bei der Verdünnung von 1/5 negativ reagierenden Tiere als „Null" gerechnet werden, so zeigt sich, dass die subkutane Immunisierung den niedrigsten Durchschnittswert ergibt. Der Durchschnitt der pulmonal immunisierten Gruppe ist ungefähr doppelt so gross, aber der Durchschnittswert der intraperitoneal immunisierten Gruppe ist noch wesentlich höher.

Ungefähr das gleiche Bild ergibt sich auch bei der folgenden Serie, die 15 Tage nach Immunisierung untersucht wurde. Hier werden sogar Titer von 1/2560 und einmal sogar von 1/5120 erreicht. Die Berechnung des Durchschnittes ergibt auch hier wieder den niedrigsten Wert bei der subkutanen Immunisierung, während die pulmonale einen nicht ganz doppelt so hohen Durchschnittswert ergibt. Am besten ist auch hier wieder der Durchschnittswert bei intraperitonealer Immunisierung.

Tabelle 2.

Einzeltiter und Durchschnitt der Antikörper gegen Vaccine-Haemagglutination beim Meerschweinchen nach Vorbehandlung mit Karbouwenvirus.

Intervall in Tagen	Vorbehandlung mit 0,5 cc 1/10 Virus-Suspension:		
	Subcutan	Intraperitoneal	Pulmonal
5	1/5 = 0	1/5 = 0	1/5 = 0
	1/5 = 0	1/5 = 0	1/5 = 0
	1/5 = 0	1/5 = 0	1/5 = 0
	1/5 = 0	1/5 = 0	1/5 = 0
		1/5 = 0	1/5 = 0
7 + 8	1/5 = 0	1/5 = 0	1/5 = 0
	1/5 = 0	1/5 = 0	1/5 = 0
	1/5 = 0	20	1/5 = 0
	1/5 = 0	40	1/5 = 0
	1/5 = 0	40	1/5 = 0
	1/5 = 0	40	10
	5	40	10
	20	80	20
	20	80	20
	40	160	80
	40	160	160
	40	160	160
	40	320	160
	160	320	320
	160	640	
		640	
	Durchschnitt=35	Durchschnitt=171	Durchschnitt=67
15	80	5	20
	80	80	40
	160	160	80
	160	320	160
	320	640	320
	320	1280	320
	640	1280	320
	640	1280	640
	640	2560	640
	1280	2560	1280
	2560	2560	2560
		2560	2560
		2560	5120
	Durchschnitt=625	Durchschnitt =1373	Durchschnitt =1082

Aus diesen Versuchen geht hervor, dass beim Meerschweinchen 5 Tage nach Immunisierung noch keine Antistoffe nachweisbar waren, dass aber schon 2—3 Tage später verhältnissmässig hohe Titer vorhanden waren. Eine pulmonale Immunisierung lieferte in diesen Versuchen einen höheren Durchschnittstiter als die subcutane Immunisierung. Noch besser aber war beim Meerschweinchen in diesen Versuchen die intraperitoneale Immunisierung.

Versuche an bunten Ratten.

In der Tabelle 3 ist zunächst einmalige und zweimalige intraperitoneale Immunisierung zusammengestellt. Es zeigt sich, dass bei Verwendung der sehr hohen Dosis von 2,0 ccm der Verdünnung 1/10 kein Unterschied in der Antistoffbildung zu ersehen ist. Die Titer gehen von 1/80 bis 1/2560.

Ferner finden sich in der Tabelle die Titerbestimmungen von 24 normalen Ratten. 16 davon weisen keinen Titer in der Verdünnung von 1/5 auf, 4 aber solchen von 1/5, 2 von 1/10 und 2 von 1/20. Normale bunte Ratten können also einen schwachen Gehalt an Vaccinevirus-Haemagglutination hemmenden Antistoffen besitzen.

Von besonderem Interesse ist aber Tabelle 4, in der 13 Einzelversuche zusammengestellt sind. Es wurde jeweils eine Serie bunter Ratten in 3 bzw. 2 Gruppen verteilt und mit 0,1 ccm einer Virusverdünnung von 1/10 subcutan, intraperitoneal und pulmonal bzw. subcutan und pulmonal immunisiert.

Bei der Untersuchung nach 4 Tagen finden sich entweder keine oder nur so niedrige Titer, wie man sie auch bei unbehandelten Kontrollratten beobachten kann. Aber schon nach 6 Tagen erhält man Titer bis zu 1/640. Schliesslich ergeben sich später Titerwerte bis zu 1/40960 bei pulmonal immunisierten Ratten.

Sieht man von den ersten 3 Serien ab, die nach 4 und 6 Tagen untersucht wurden, so zeigt sich bei allen anderen 10 Serien mit grosser Deutlichkeit, dass die pulmonale Immunisierung erheblich besser ist als die subcutane. Die Durchschnittswerte der pulmonalen Gruppen sind ca. 3 bis 10 mal so hoch als die entsprechenden der subcutanen. Ganz im Gegensatz zu den Versuchen am Meerschweinchen erweist sich hier bei der bunten Ratte auch die intraperitoneale Immunisierung deutlich der pulmonalen unterlegen. Vergleicht man die intraperitoneale mit der subcutanen Immunisierung, so ist in einzelnen Versuchen die subcutane und in anderen die intraperitoneale besser.

Tabelle 3.

Antistoffe gegen Vaccinevirus-Haemagglutination bei schwarz-weissen Ratten.

Immunisierung	Intervall in Tagen	Anzahl der Tiere mit Serumtiter von:											
		1/5=0	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	Total
1×2,0 1/10 ip.	14–24						4	4	6	10	5	2	31
2×2,0 1/10 ip.	20						5	4	9	8	11	1	38
Normale Ratten	—	16	4	2	2								

Tabelle 4.

Einzeltiter und Durchschnitt der Antikörper gegen Vaccine-Haemagglutination bei schwarz-weissen Ratten nach Vorbehandlung mit Karbouwen-virus

Vorbehandlung mit 0,1 cc. 1/10 Virus-Suspension:

Tage Intervall	Subcutan		Intraperitoneal		Pulmonal	
	Einzeltiter	Durchschnitt	Einzeltiter	Durchschnitt	Einzeltiter	Durchschnitt
4	5, 5, 5, 1/5=0, 1/5=0, 1/5=0	2,5	1/5=0, 1/5=0, 1/5=0, 1/5=0, 1/5=0, 1/5=0, 1/5=0, 1/5=0	0	5, 5, 10, 1/5=0, 1/5=0, 1/5=0, 1/5=0	2,8
6	10, 10, 10, 10, 10, 10, 20, 40, 160	31			10, 10, 10, 40, 80, 80, 80, 160	59
6	5, 10, 20, 80, 320, 320	125	1/5=0, 20, 40, 80, 320, 640	183	10, 20, 40, 80, 80, 640	145
7	10, 10, 10, 10, 10, 10, 10, 40, 160, 160	43			10, 20, 40, 40, 40, 160, 320, 320	119
8	5, 5, 5, 20, 40, 40, 40, 40, 40, 160, 320, 2560	273	5, 5, 5, 5, 20, 40, 40, 40, 320, 320	80	640, 640, 1280, 1280, 1280, 1280, 1280, 1280, 2560, 2560	1408
8	20, 160, 160, 160, 1280, 2560, 2560, 2560, 5120, 5120	1970	40, 40, 160, 320, 320, 640, 640, 640, 1280, 5120	820	160, 1280, 5120, 5120, 5120, 5120, 5120, 10240, 10240, 10240	5726
8	20, 20, 40, 40, 40, 320, 320, 320, 1280, 2560	496			80, 320, 1280, 1280, 2560, 2560, 2560, 2560, 5120, 5120	2344
9	5, 5, 20, 20, 20, 80, 80, 160, 160, 2560	311	5, 5, 5, 5, 20, 80, 160, 160, 640, 5120	620	20, 40, 80, 320, 640, 5120, 5120, 5120, 10240, 10240	3694
9	320, 1280, 1280, 1280, 1280, 2560, 2560, 2560, 2560, 5120, 5120, 10240, 10240, 20480	4777			1280, 2560, 5120, 5120, 5120, 5120, 10240, 20480, 20480, 40960, 40960, 40960, 40960, 40960	20023
15	320, 640, 640, 640, 640	574	320, 320, 640, 1280, 2560	1024	1280, 1280, 5120, 5120	4608
15	320, 320, 320, 320, 640, 640, 640, 640, 640, 640, 5120	960	160, 160, 320, 320, 640, 640, 1280, 2560	760	320, 1280, 1280, 1280, 5120, 5120, 10240	3520
16	20, 160, 160, 320, 320, 640, 2560	597	40, 80, 80, 320, 320, 640, 640	303	1280, 2560, 2560, 5120, 5120, 10240, 20480	6766
16	40, 80, 320, 320, 640, 640, 2560	657	40, 320, 320, 320, 640, 1280	487	1280, 2560, 2560, 2560, 10240	3840

Tabelle 5.

Einzeltiter und Durchschnitt der Antikörper gegen Vaccine-Haemagglutination unter Benutzung der 4× und 2× agglutinierenden Virusdosis.

Immunisierung	Agglutinierende Dosis 4×	Agglutinierende Dosis 2×
Subcutan:	20	320
	20	1280
	20	1280
	20	1280
	40	1280
	80	2560
	80	2560
	160	2560
	160	2560
	320	5120
	320	5120
	640	10240
	1280	10240
	2560	20480
	Durchschnitt = 409	Durchschnitt = 4760
Pulmonal:	80	1280
	160	2560
	320	5120
	320	5120
	1280	5120
	1280	5120
	1280	10240
	2560	20480
	2560	20480
	2560	40960
	2560	40960
	2560	40960
	2560	40960
	5120	40960
	Durchschnitt = 1800	Durchschnitt = 20023

Schliesslich ist in der Tabelle 5 von Versuch 9 der Tabelle 4 nebeneinander die Titer der agglutinationshemmenden Antistoffe für die doppelte und für die vierfache gerade noch haemagglutinierende Virusdosis gegenübergestellt. Es zeigt sich, dass das Verhältnis der Durchschnittswerte bei den subcutan und pulmonal immunisierten Ratten ungefähr in der gleichen Grössenordnung liegt.

Besprechung der Ergebnisse.

Bemerkenswert ist die Tatsache, dass bei Ratten nach pulmonaler Immunisierung stärkere humorale Antistoffe gegen die Vaccinevirus-Haemagglutinine entstehen, als nach subcutaner oder intraperitonealer Einverleibung des Antigens. Bei der in diesen Versuche angewandten Immunisierungstechnik handelt es sich aber nicht nur um eine ausschliessliche Einverleibung des Materials in die Lunge, auch die Nasenschleimhaut und die Schleimhaut der übrigen Luftwege kommt in Kontakt mit dem Virus.

Immunisierung der oberen Luftwege und der Lungen ist verschiedentlich versucht worden. Nachdem Dzierzgowski (5) und Blumenau (2) Antitoxinbildung nachgewiesen hatten, wenn sie Diphtherietoxin in Wattetampons auf die Nasenschleimhaut gebracht hatten, berichtete Sanarelli (10) über nasale Immunisierung gegen Typhusbacillen, Choleravibrionen und andere Bakterien.

Eine intratracheale Immunisierung gegen Tuberkulose versuchten Besnoit, Leclainche und Morel (1) bei Rindern. Russel und Steffen (9) immunisierten auf diese Weise Affen gegen Pneumokokken und Schewelff (11) Hunde gegen Diphtherietoxin.

Durch direkte Injektion in die Lungen suchten Blumenthal (3) und Kaneko (8) Pferde gegen Diphtherietoxin zu immunisieren.

Eine Immunisierung aller Atmungsorgane gemeinsam erfolgt bei Inhalation versprayten Materials und bei nasaler Installation von Antigen unter Aethernarkose. Bei der Inhalation, die von verschiedenen Autoren angewandt wurde, ist wegen der relativ geringen Antigenmenge die entstehende Immunität nicht selten unzureichend. Die nasale Instillation unter Aethernarkose ergibt dagegen recht befriedigende Resultate. So fand Collier (4) nach derartiger Immunisierung mit Pneumokokken dass nicht nur eine lokale, sondern auch eine allgemeine Immunität gegen intraperitoneale Infektion entstand.

Schon im alten China immunisierte man gegen Pocken durch das Einblasen staubfein gemahlener getrockneter Krusten in die Nase. Durch tracheale Injektion von Vaccine immunisierte Haaland (1905) erfolgreich Kaninchen. Nach Gins (6) lassen sich Kaninchen durch Inhalation verstäubter Vaccine vollkommen immunisieren. Gleichfalls kann man nach Gins Schafe durch Inhalation von Vaccinevirus gegen eine nachfolgende schwere Infektion mit Schafpocken schutzen. Gyuere vaccinierte und revaccinierte Kinder

nasal. Durch nasale Einverleibung von stark verdünnter Vaccine entwickelte sich bei Säuglingen eine milde, schnell wieder verschwindende Rhinitis und etwas Fieber. Hierauf trat schnell Immunität auf. Die nasale Revaccination erzeugte Schwellung der Mucosa, auch Auftreten von Papeln oder Vesikeln. Die Immunität wurde verstärkt. Ein Nachlassen des „Impfschutzes" wurde früher angezeigt als durch cutane Revaccination.

Aus den hier mitgeteilten Versuchen geht hervor, dass sich auch Meerschweinchen und Ratten pulmonal unter Aethernarkose sehr gut mit Vaccine immunisieren lassen. Hierdurch entstehen humoral nachweisbare Antistoffe gegen die Vaccinevirus-Haemagglutination und zwar sind bei Meerschweinchen die Titer dieser Antistoffe etwas höher als nach subcutaner Vorbehandlung mit der gleichen Menge lebender Vaccine, während intraperitoneale Immunisierung noch bessere Ergebnisse liefert. Bei Ratten werden aber nach pulmonaler Immunisierung ganz erheblich höhere Titer der Anti-Haemagglutinine erzielt, als dies nach subcutaner oder intraperitonealer Vorbehandlung der Fall ist.

Die hohen Antistofftiter gegen die Virus-Haemagglutinine bei Meerschweinchen und Ratte (1/40.000) stehen in schroffem Gegensatz zu den niedrigen Titern (ca. 1/160 usw.) beim cutan oder intravenös immunisierten Kaninchen. Sie sind auch deutlich höher als die Titer des Menschen nach Revaccination, die nur ausnahmsweise 1/1280 erreichen und in der Regel viel niedriger liegen. Seren von Variolapatiënten erreichen zwar höhere Titer, reichen aber doch nicht an die der Rattensera heran. Ein gewisses Analogon findet sich nur bei den menschlichen Influenzaseren, bei denen bei der Haemagglutination auch so extrem hohe Titer gefunden werden.

Die Frage, warum nach pulmonaler Immunisierung höhere Antistoffwerte erreicht werden als nach subcutaner oder intraperitonealer, lässt sich schwer beantworten. Man muss wohl annehmen, dass die Atemwege bei Pocken die natürliche porte d'entré darstellen, wo optimale Bedingungen für das Haften und die ersten Entwicklung des eingedrungenen Virus gegeben sind. Bei der nachfolgenden Allgemeininfektion gelangen daher jedenfalls grössere Virusmengen in den Blutumlauf und damit zu den antistoffbildenden Zellsystemen. Diese günstigen Bedingungen liegen besonders bei der Ratte bei subcutaner oder intraperitonealer Infektion offenbar nicht im gleichen Masse vor.

Aus dem zwar hohen Virusgehalt aber niedrigem Haemagglutiningehalt der oberflächlichen Lagen der Kaninchenhaut hatten Stone und Burnet (12) geschlossen, dass das Haemagglutinin vorwiegend in den Epithelcellen gebildet, dann aber oxydiert und zerstört wird

In ähnlicher Weise möchte man annehmen, dass sich das Haemagglutinin nach pulmonaler Infektion in den Lungenepithelien bildet, aber noch vor erfolgter Oxydation infolge der starken Circulation in den Lungenalveolen in die Blutbahn übergeht. Auf diese Weise käme reichlicher aktives Haemagglutinin als nach subcutaner Infektion an die Antistoffe bildenden Zellsysteme heran, falls nicht schon teilweise im Lungengewebe die Antistoffbildung vor sich ginge. Es wäre in diesem Falle nur noch die Diskrepanz zwischen dem hohen Agglutiningehalt im Epithelmaterial des Karbouws und dem niedrigen des Kaninchens zu erklären.

Zusammenfassung.

Meerschweinchen und bunte Ratten bilden nach Immunisierung mit Vaccinevirus vom Karbouw humorale Antistoffe gegen die Vaccinevirus-Haemagglutination. Bei pulmonaler Immunisierung unter Aethernarkose sind beim Meerschweinchen diese Antistoffe etwas höher als nach subcutaner Einverleibung der gleichen Virusmenge, bei der weissen Ratte aber ganz erheblich höher als nach subcutaner und intraperitonealer Vorbehandlung.

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THE INFLUENCE OF SULPHITE ON THE METHANE FERMENTATION OF SODIUM AND CALCIUM ACETATES

by

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§ 1. Introduction.

In the manufacture of certain qualities of card-board from straw in Holland monosulphite is used as a digesting agent. Hence the waste water from the respective straw-board mills contains some sulphite, which appears to interfere with methane production from the straw-board waste water (*i.e.* the waste water from the ordinary straw-board process in which lime is used as a digesting agent, which process is applied by these mills side by side with the monosulphite process).

This phenomenon suggests a detrimental action of sulphite on the micro-organisms active in methane production and it was thought desirable to start an investigation on the influence of sulphite on methane fermentation. The work was begun in 1944 in the Laboratory for Microbiology of the Technical University at Delft, continued at the Microbiological Division of the Agricultural Experiment Station and Institute for Soil Research T.N.O. at Groningen in 1947 and concluded at the Straw Station's new laboratory in 1948. The authors are greatly indebted to Prof. Dr A. J. Kluyver and Dr F. C. Gerretsen, respective Directors of the two former institutes, for the hospitality enjoyed in their laboratories.

§ 2. The culture of methane bacteria.

Enrichment cultures of methane bacteria were obtained as follows. A layer of mud from a sewer or from a waste water pond of a straw-board mill was put into stoppered 250 ml bottles which were then filled with a solution of the following composition:

sodium acetate	1 %
NH_4Cl	0.1 %
K_2HPO_4	0.05 %
$MgSO_4$	0.01 %

In order to exclude the oxygen from the air as much as possible, the medium was boiled and quickly cooled and a few drops of 10 % solution of Na_2S. 9 aq per l were added before use.

The enrichment cultures contained about 100 ml of mud per 250 ml bottle and were incubated at 30° C. When a vigorous fermentation had been obtained 5 ml of the mud were transferred to a bottle containing the same medium and a quantity of shredded asbestos as an artificial mechanical support for the bacteria (cf. COOLHAAS (6), BREDEN and BUSWELL (2)). Commercial white asbestos, treated with aqua regia and subsequently washed with water, was employed. The subcultures obtained in this way were transferred several times, so that finally cultures free from any mud were obtained. For each transfer about 5 ml of the asbestos sediment carrying the bacteria were used.

After having obtained a sufficient number of purified enrichment cultures, they were maintained by siphoning off the supernatant liquid and replacing it by fresh medium as soon as gas production ceased. The methane produced was collected in calibrated tubes above dilute NaOH solution. The cultures were incubated at 30° C.

§ 3. Influence of the composition of the medium on the methane fermentation of sodium acetate.

The actual investigation of the action of sodium sulphite on methane fermentation was preceeded by some preliminary experiments to ascertain the influence of the composition of the medium on fermentation.

In the first place the influence of the acetate concentration upon the rate of methane production was investigated. For this purpose a series of cultures was started with $^1/_4$, $^1/_2$ and 1 % sodium acetate, respectively. Fig. 1 shows that the acetate concentration has considerable influence on the rate of gas production. The culture with $^1/_2$ and especially that with $^1/_4$ % Na-acetate, had a considerably lower rate of fermentation. As appears from the areas between the curves and the abscissa, the total quantity of methane produced from the acetate is about proportional to its concentration.

When, on the other hand, the quantity of asbestos was decreased

to ½ or ¼, the rate of fermentation remained the same.

Apparently a quantity of inert sediment of 6 to 7 g per bottle of ca. 250 ml ensured maximum fermentation. Therefore, all further cultures were started with this quantity of asbestos. Whereas the quantity of inert sediment therefore proved to be of no great

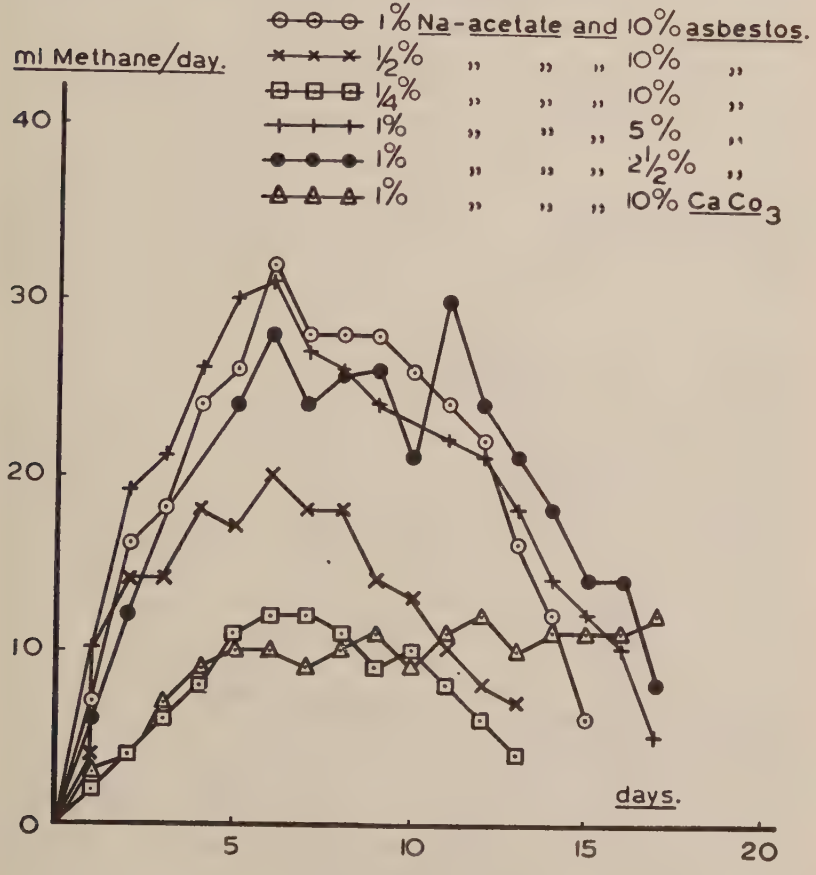

Fig. 1.

significance, the quality appeared to be very important: substitution of ground chalk for the asbestos resulted in a much lower rate of fermentation. This may be explained in two ways. In the first place the medium with chalk has a higher pH than that with asbestos, a pH of 6.0 to 6.5 being the optimum for methane fermentation (see below). A second explanation may be that the chalk particles may be unsuitable as a mechanical support of the bacteria.

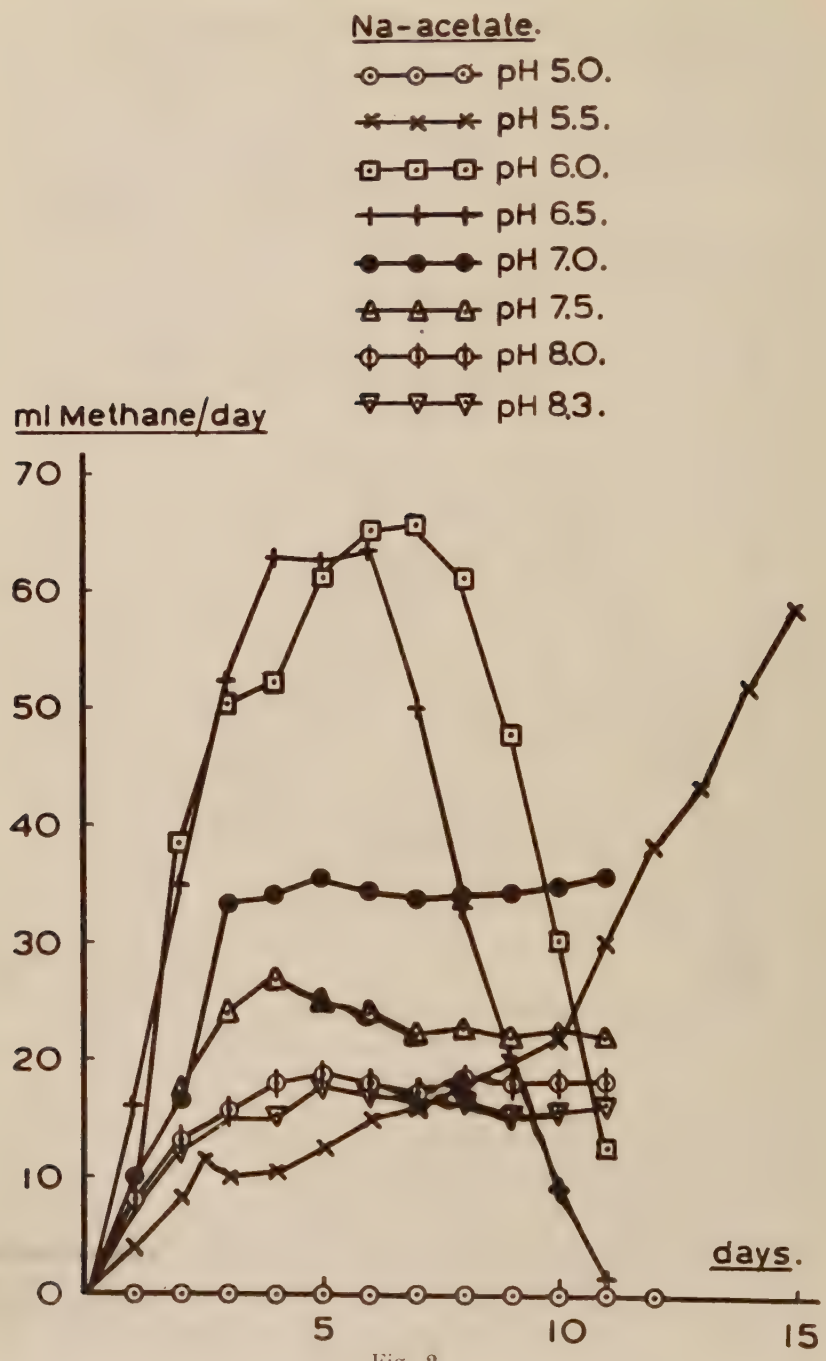
Na-acetate.
pH 5.0.
pH 5.5.
pH 6.0.
pH 6.5.
pH 7.0.
pH 7.5.
pH 8.0.
pH 8.3.
ml Methane/day
70
60
50
40
30
20
10
0
5
10
15
days.

Fig. 2.

The pH of the medium has considerable influence on methane fermentation. This is evident from fig. 2. In these cultures the medium was buffered by mixtures of secundary sodium and primary potassium phosphate in a total concentration of $^1/_{15}$ molar. For the pH's below 6.5 part of the sodium acetate had to be replaced by an equivalent amount of acetic acid [1]).

The amounts of phosphate, acetate and acetic acid required, expressed in g/l, are given below.

pH	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.3
KH_2PO_4	9.00	8.72	7.90	6.17	3.54	1.36	0.23	—
Na_2HPO_4	0.12	0.48	1.54	3.8	7.24	10.1	11.6	11.87
sodium acetate	7.05	8.85	9.6	10	10	10	10	10
acetic acid	1.30	0.51	0.17[5]	—	—	—	—	—

The pH was checked with Lyphan paper. Buffering the medium is advisable on account of the tendency of the pH to rise during fermentation by substitution of bicarbonate for acetate ions. Thus, the pH soon rises to 7.5—8.5 in an unbuffered medium [2]). With the buffers mentioned above this shift in pH, though not prevented completely, is much smaller, being highest in the media with additional acetic acid: in one case a culture with an initial pH of 5.5 changed to 6.6 at the end of the fermentation. This is due to the substitution of a bicarbonate/carbonic acid for the initial acetate/acetic buffer, the former system, moreover, losing CO_2 escaping with the CH_4.

It appears from fig. 2 that the highest fermentation rate occurred at a pH of 6.0 to 6.5 [3]). This optimum is in agreement with the results of CLARK and ADAMS (5) who also found the rate of methane production from municipal sewage sludge to be higher at 6.0—6.5 than at 7.0.

The course of the fermentation in the medium buffered at 5.5

[1]) The composition of the sodium acetate preparation employed was $CH_3COONa \cdot 3H_2O$.

[2]) From the two dissociation constants of carbonic acid (see G. N. LEWIS and M. RANDALL, Thermodynamics. New York 1923, p. 577—578) the pH of a solution of $NaHCO_3$ equivalent to 1% Na-acetate can be calculated as 8.4 at 25°C.

[3]) The drop of the rate after 6—7 days is due to exhaustion of the acetate in the medium.

clearly demonstrates the above-mentioned shift in pH. The initial rate was small but increased gradually to that of a culture started at 6.0 or 6.5.

At 5.0 no fermentation occurred at all. This may be due to a toxic action of the undissociated acetic acid present. (cf. also BUSWELL and co-workers (3,4,8)). Preliminary experiments showed that the methane-producing bacteria are not killed by the action of buffers of pH's 4.5 and 5.0: when the media containing these acid buffers were replaced by a medium buffered at 7.0, fermentation started again which shows that inhibition by a low pH is reversible.

§ 4. INFLUENCE OF SODIUM SULPHITE UPON METHANE FERMENTATION OF SODIUM ACETATE.

The influence of sodium sulphite upon the rate of methane fermentation was investigated with series of cultures to which increasing quantities of sulphite had been added.

In strongly active cultures the liquor over the asbestos layer was replaced by a fresh medium with a certain percentage of Na_2SO_3. Later on the Na_2SO_3 was sometimes added the day after the renewal of the medium. This procedure was only followed when there was some doubt whether all these cultures originally had the same rate of fermentation. The gas production during the first day after renewal then served as a check on the homogeneity of the series in this respect. With a view to the autoxidizability of sulphites the concentration of the stock solution was determined iodometrically.

In the beginning unbuffered cultures with an initial pH of about 7.0 were used. The results of one of these experiments are shown in fig. 3. It appeared that a concentration of only 0.01 % Na_2SO_3 had practically no effect. On the other hand, a concentration of 0.05 % was already distinctly inhibitory. With increasing concentration inhibition became gradually stronger. At 0.25 % Na_2SO_3 fermentation was inhibited completely during the first six days, whereupon a slight recovery occurred. The cultures with 0.07 % and 0.1 % Na_2SO_3 show the same phenomenon in a somewhat slighter degree: a relatively strong inhibition during the first days followed by a recovery.

This shows that the methane bacteria are not killed by the sulphite, in other words, Na_2SO_3 has a bacteriostatic rather than

a bactericidal action. This is shown still more clearly in an experiment in which fermentation had been stopped altogether by the addition of 0.5 % Na_2SO_3. When some days afterwards the super-

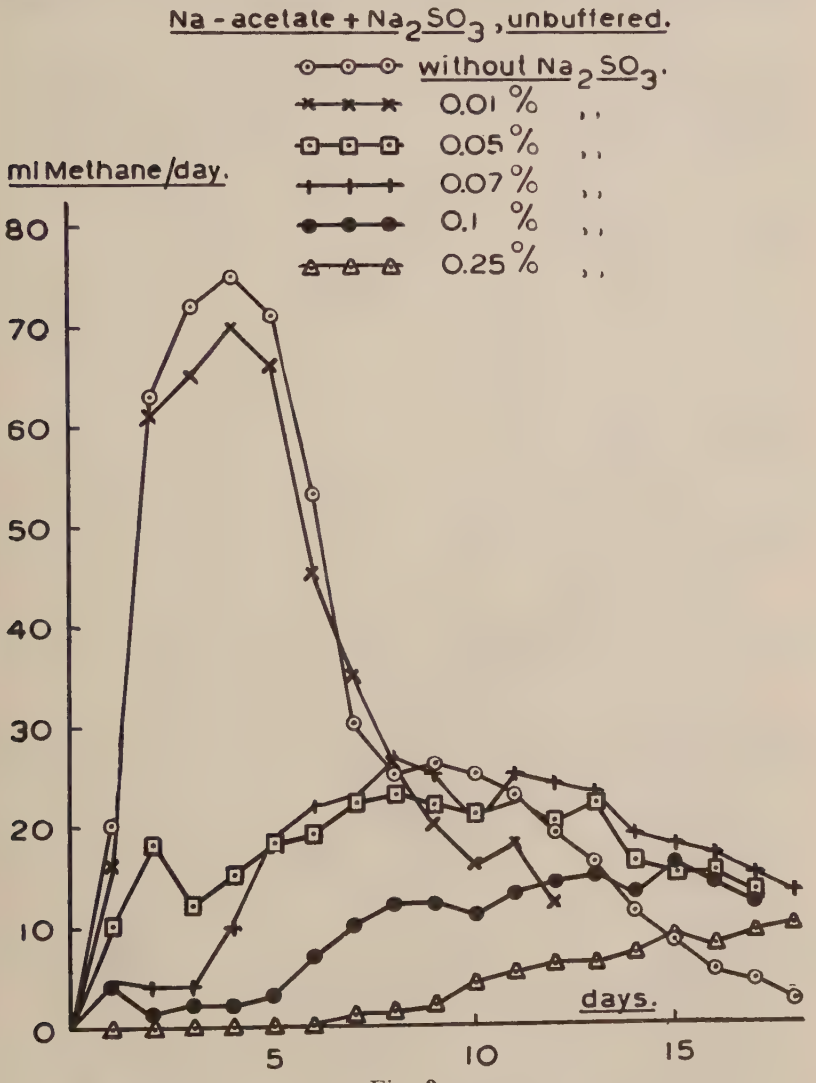

Fig. 3.

natant liquor was replaced by the medium without Na_2SO_3, fermentation was resumed. In this connection the question may be raised whether the resumption of methane fermentation which,

as we have observed, occurred spontaneously in cultures with 0.25 % Na_2SO_3 or less, is due to a disappearance of sulphite from the medium owing to the activity of sulphate-reducing bacteria. This possibility was not investigated, but seems quite probable

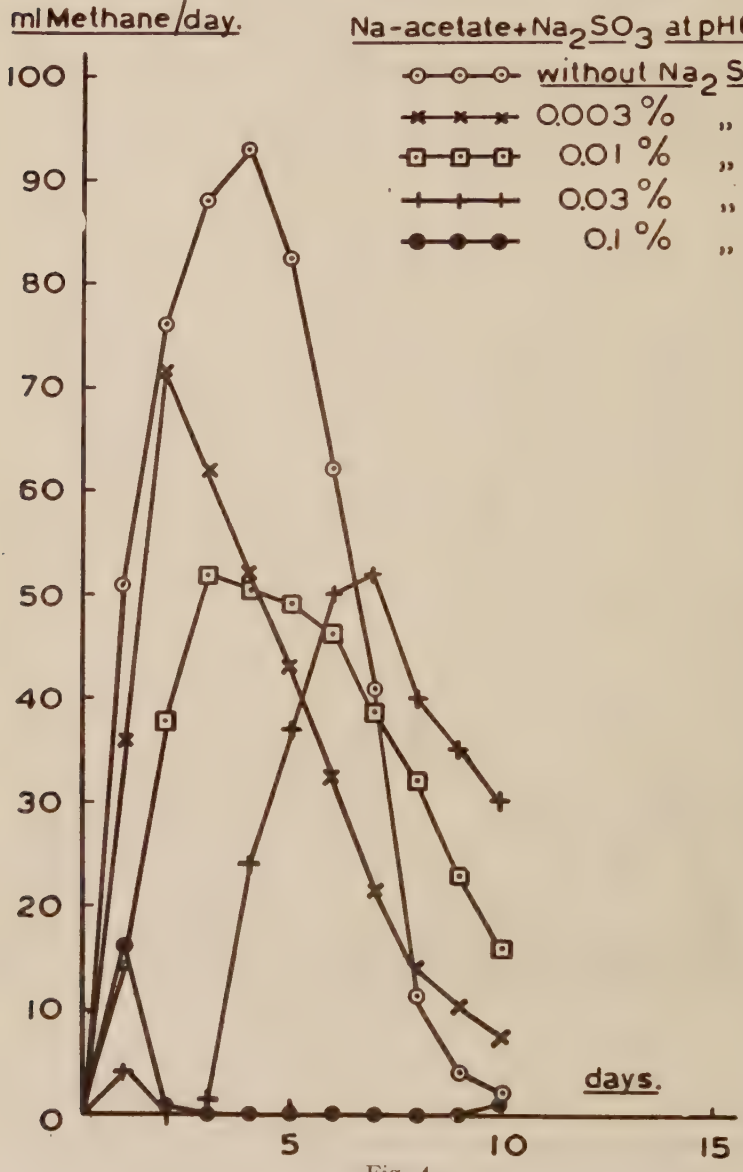

Fig. 4.

if we take into account the occurrence side by side of methane and sulphate-reducing bacteria in nature, and the ability of the latter to hydrogenate sulphite to hydrogen suphide which is quite harmless to the former bacteria.

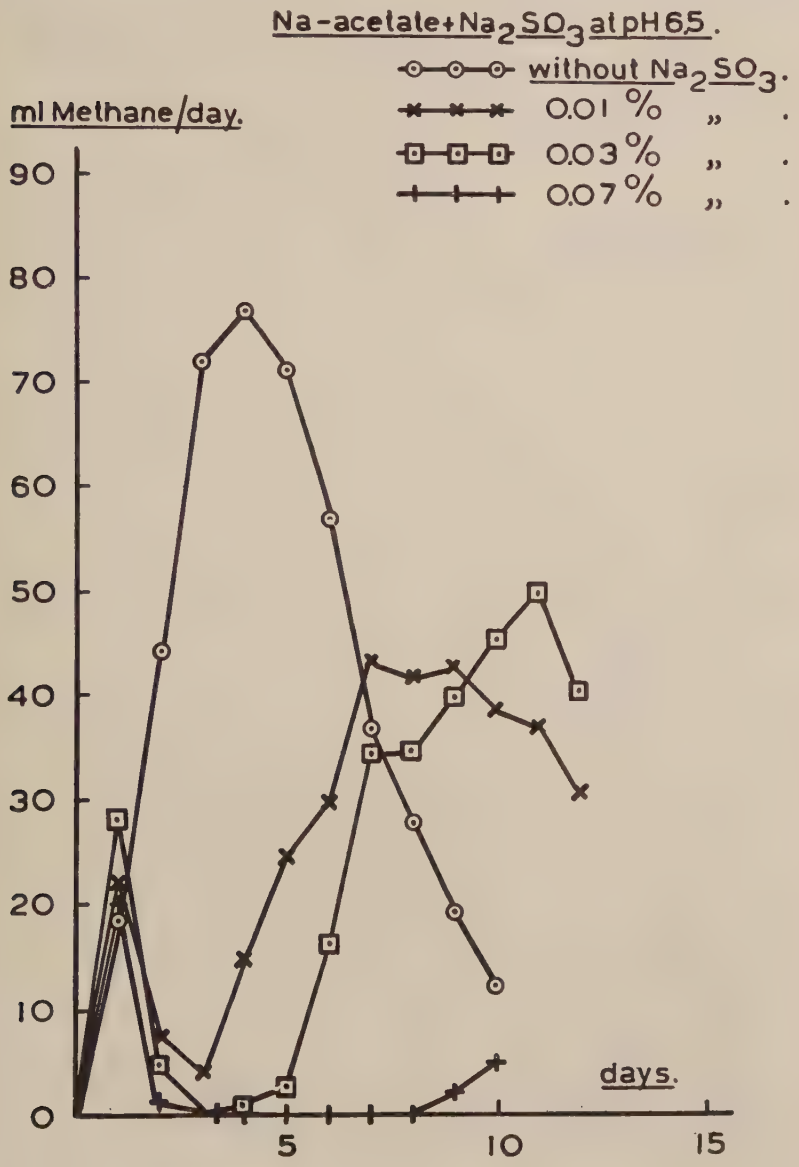

Fig. 5.

The inhibitory action of Na_2SO_3 was not only investigated as a function of its concentration, but also in dependence on the pH. For this purpose the same buffers were used as mentioned in § 3. The results of these experiments are presented in figures 4, 5, 6 and 7. In figure 4, showing the relation between rate of fermen-

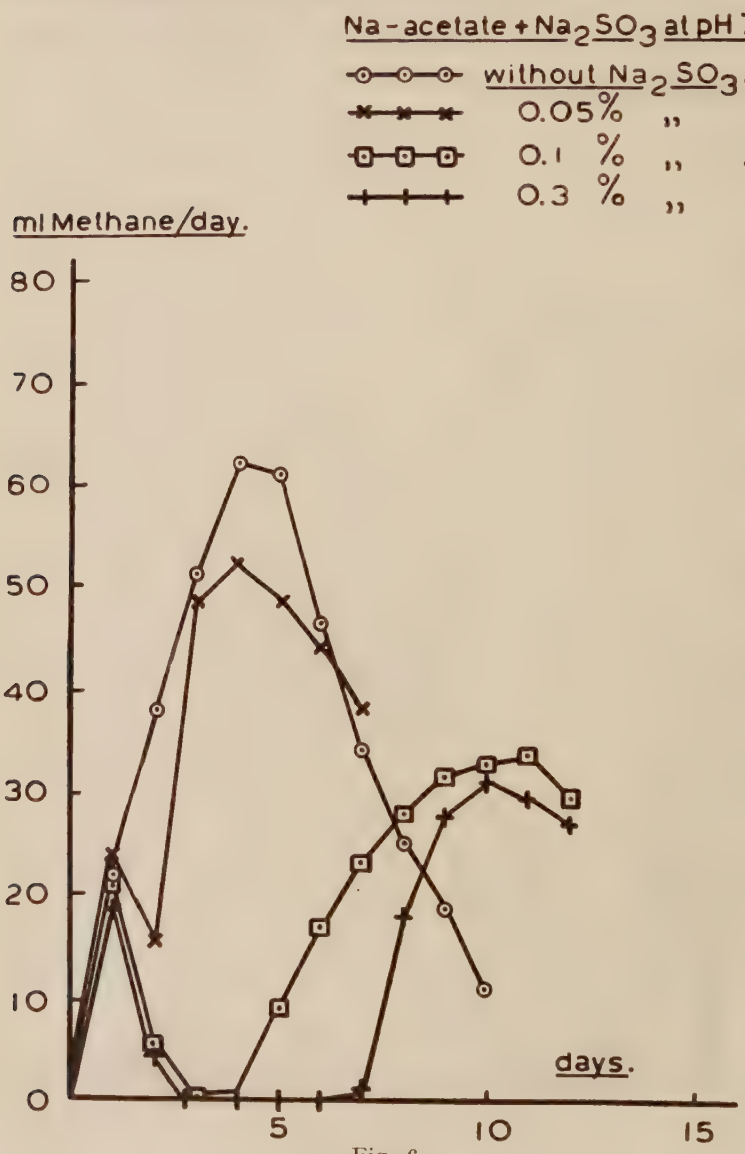

Fig. 6.

tation and concentration of Na_2SO_3 at pH 6.0, a perceptible inhibition is noted with as little as 0.003 % Na_2SO_3. A concentration of 0.03 % caused a strong initial inhibition which was followed, however, by a rather spectacular recovery. A concentration of 0.1 % Na_2SO_3 inhibited fermentation completely for several days. However, methane production started again after 3 weeks. The

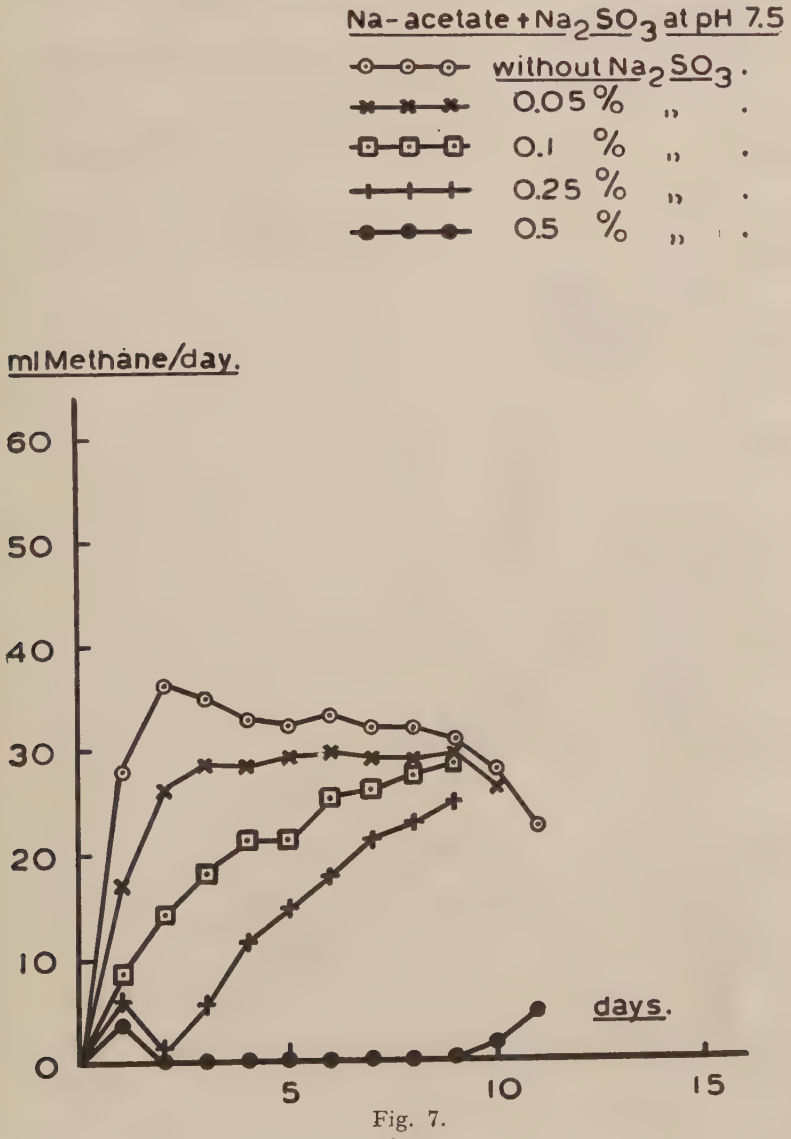

Fig. 7.

series of cultures at a pH of 6.5 (fig. 5) shows about the same picture, a concentration of 0.07 % Na_2SO_3 inhibited methane production completely for 8 days. At a pH of 7.0 (fig. 6) the concentration of Na_2SO_3 required for a similar inhibition is about three times, and at a pH of 7.5 (fig. 7) even about five times higher than at 6.5.

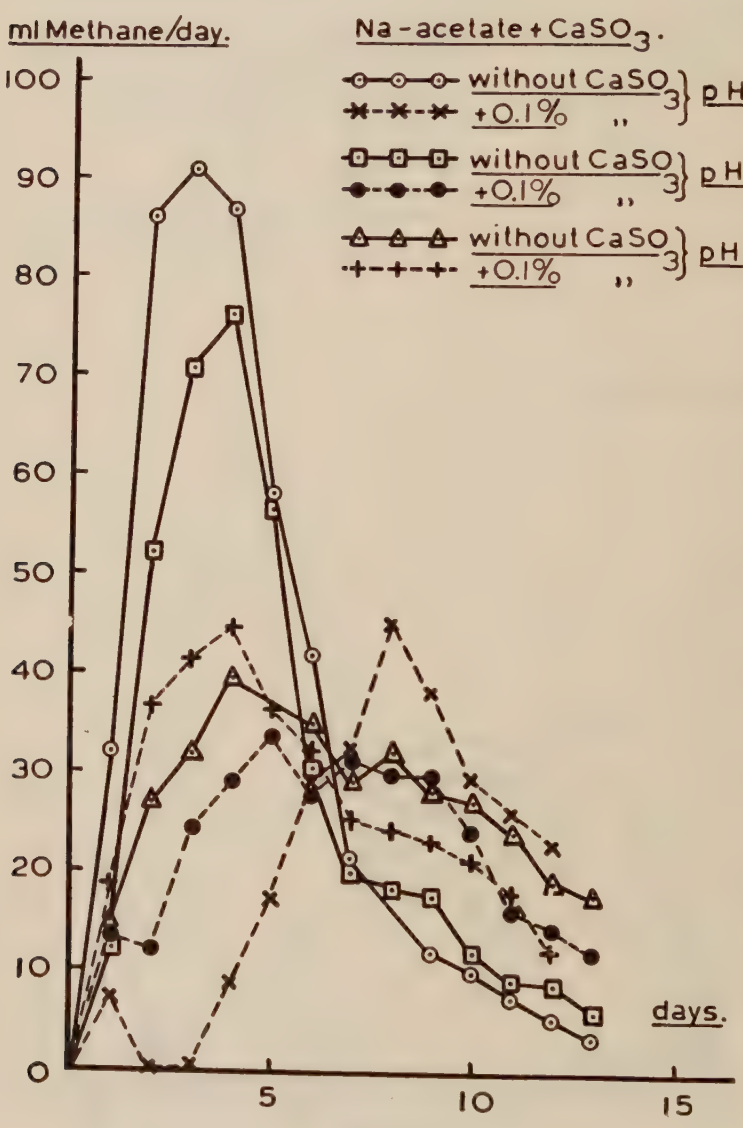

Fig. 8.

The usually slow development of the inhibition can be explained by the fact that the active bacteria are present in the asbestos layer, whereas the sulphite is originally only present in the supernatant liquid.

§ 5. Influence of calcium sulphite on methane fermentation of sodium acetate.

After it had appeared from the preceding experiment that methane fermentation was inhibited by Na_2SO_3, we tried to find a means of eliminating this detrimental action. In the first place precipitation of the sulphite with calcium ions was considered, the solubility of $CaSO_3$ amounting to only 43 mg/l [1]).

Therefore, it was deemed advisable to examine the action of this substance on methane production. For this purpose cultures were used buffered with phosphate at pH's of 6.5, 7.0 and 7.5 to which solid $CaSO_3$ was added in amounts corresponding to 0.1 %, in comparison with blank cultures of the same pH's. The SO_3'' content of the $CaSO_3$ used was again checked by iodometric titration.

The influence of 0.1 % $CaSO_3$ on methane fermentation of sodium acetate can be read from fig. 8. In agreement with the results reported in § 3 the blank culture of pH 6.5 showed the highest rate of fermentation. At a pH of 6.5 $CaSO_3$ appeared to cause a marked inhibition, the fermentation rate decreasing to zero after a few days, followed by a recovery as found before with Na_2SO_3. Also at a pH of 7.0 the addition of 0.1 % $CaSO_3$ entailed a distinct, though smaller inhibition. At a pH of 7.5 however, no inhibitory action of 0.1 % $CaSO_3$ could be observed.

Thus it appears that $CaSO_3$ has an effect similar to, though less pronounced, than that of Na_2SO_3.

§ 6. Methane fermentation of calcium acetate.

Since milk of lime is used in the manufacture of ordinary strawboard as a digesting agent, the waste liquor has a high calcium content. This prompted us to investigate the influence of sulphite on the methane fermentation also of calcium acetate. First of all it was observed that fermentation of calcium acetate in an unbuffered medium proceeded much more rapidly than that of

[1]) Handbook of Chemistry and Physics. 30th Ed. 1948, p. 395.

sodium acetate, the rate in the former case being thrice as high as that in the latter. As a medium with calcium acetate has a lower pH than one with the sodium salt, we tried to determine the in-

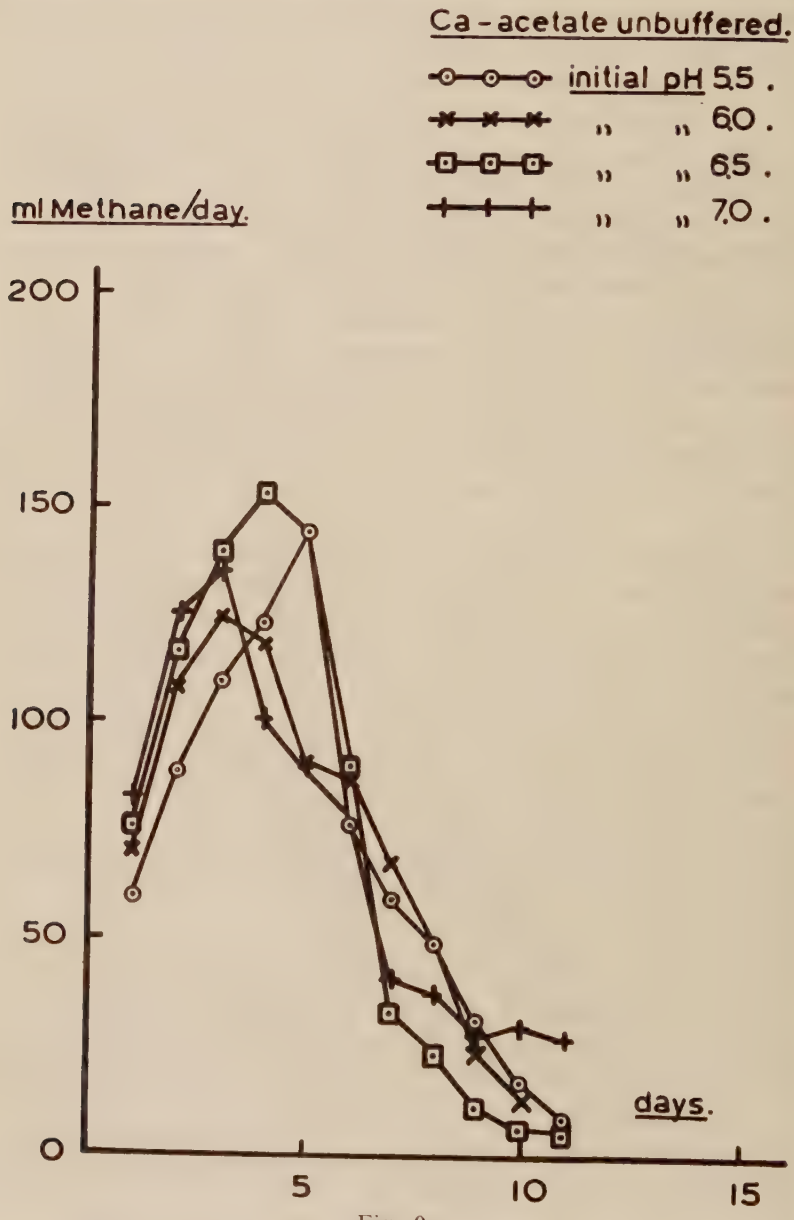

Fig. 9.

fluence of the pH upon methane fermentation of calcium acetate. However, the Ca″-ions of the calcium acetate precipitate a large proportion of the phosphate buffers, which are the only suitable buffer systems in this case.

Therefore, cultures with various initial pH's had to be prepared with unbuffered media, the pH of which was adjusted with a 1 % hydrochloric acid solution. Fig. 9 shows that a difference in initial pH in the case of an unbuffered medium has but little influence on the rate of fermentation. Only at an initial pH of 5.5 a slight inhibition in the beginning is noticeable.

Apparently the conversion of acetate/acetic acid into bicarbonate /carbonic acid soon produced a rise in pH to the more favourable level of 6.0—6.5. It appeared that in fermentations of calcium acetate this level is maintained during the whole fermentation process, whereas in unbuffered fermentations of sodium acetate the pH rises to values of 8.0 and higher. In both cases fermentation brings about a conversion of acetate into bicarbonate, but in the former the majority of the bicarbonate is converted to $CaCO_3$ and H_2CO_3 which tends to fix the pH at a value of 6.0—6.5 [1]).

Therefore, in a culture with an initial pH of 7.0 the pH decreases to about 6.3 instead of rising like it does in an unbuffered culture with Na-acetate of the same initial pH.

§ 7. Influence of sodium sulphite upon methane fermentation of calcium acetate.

For the reason outlined in § 6 the influence of Na_2SO_3 could not be investigated in buffered media. On account of the comparatively low pH of about 6.3 which is soon reached by the unbuffered cultures with Ca-acetate, one would expect a rather strong influence of Na_2SO_3. It is true that the medium contains a large excess of Ca over SO_3 ions, which might lead to precitation of the insoluble $CaSO_3$, were it not that precipitation of this salt, even from strongly supersaturated solutions is often very slow (cf. (7)) and, moreover,

[1]) From the dissociation constants of carbonic acid, the solubility product of $CaCO_3$, the sum of $[H_2CO_3]$, $[HCO_3']$, $[CO_3'']$ and $CaCO_3$ (equivalent to the acetate added as Ca-acetate) and the sum of $CaCO_3$ and $[Ca'']$ (equivalent to the Ca added as Ca-acetate), the pH, resulting from a total conversion of the acetate, can be calculated as 6.0 (ignoring loss of CO_2 escaping with the CH_4). For the final amounts of $[H_2CO_3]$, precipitated $CaCO_3$ and $[HCO_3']$ are found 0.053, 0.052 and 0.021 mol/l, respectively.

as shown by the experiments on the influence of $CaSO_3$ on fermentations of Na-acetate (vide § 5), even solid $CaSO_3$ added to a culture with a pH of 6.0—6.5 may cause appreciable inhibition.

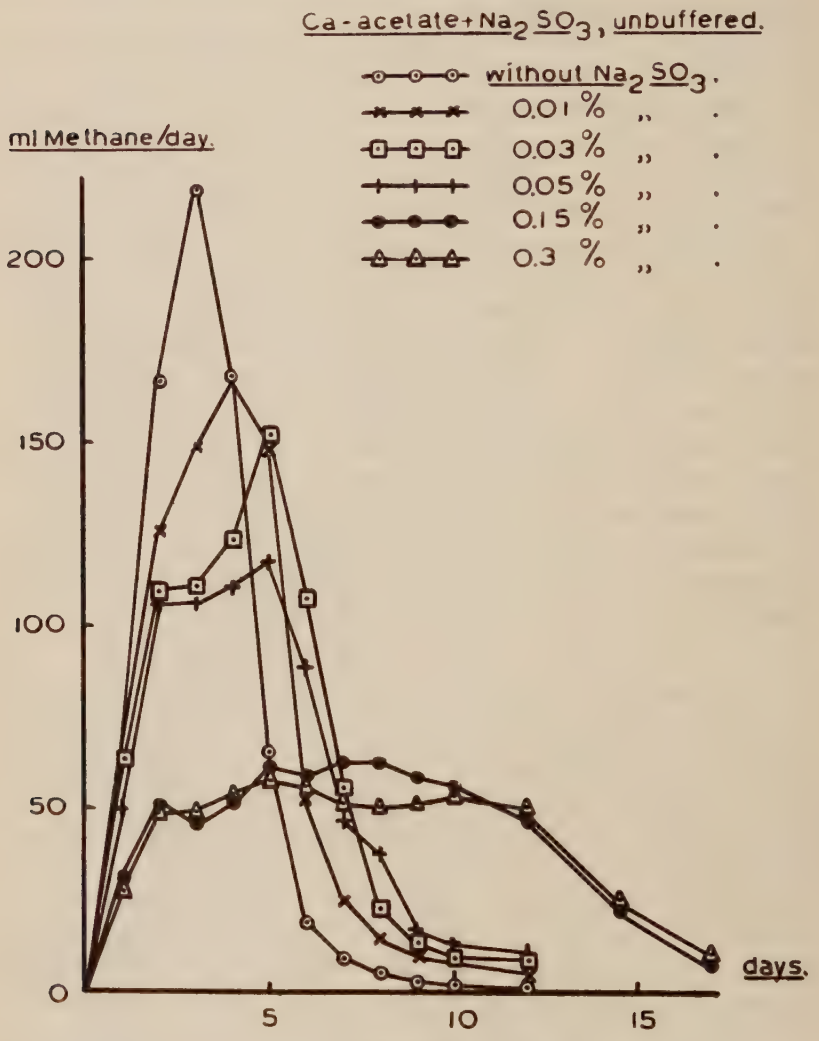

Fig. 10.

Fig. 10 shows that sodium sulphite did cause marked inhibition of the fermentation of Ca-acetate, already perceptible with 0.01 % Na_2SO_3. The inhibition gradually increased up to a concentration of about 0.15 % Na_2SO_3, 0.3 % having the same effect as 0.15 %.

It further appears that even at the highest concentrations inhibition was not complete, unlike what was found with cultures with Na acetate. Apparently some precipitation of $CaSO_3$ did occur, but it is hard to tell to what extent.

The combination of Ca-acetate and Na_2SO_3 resembles most

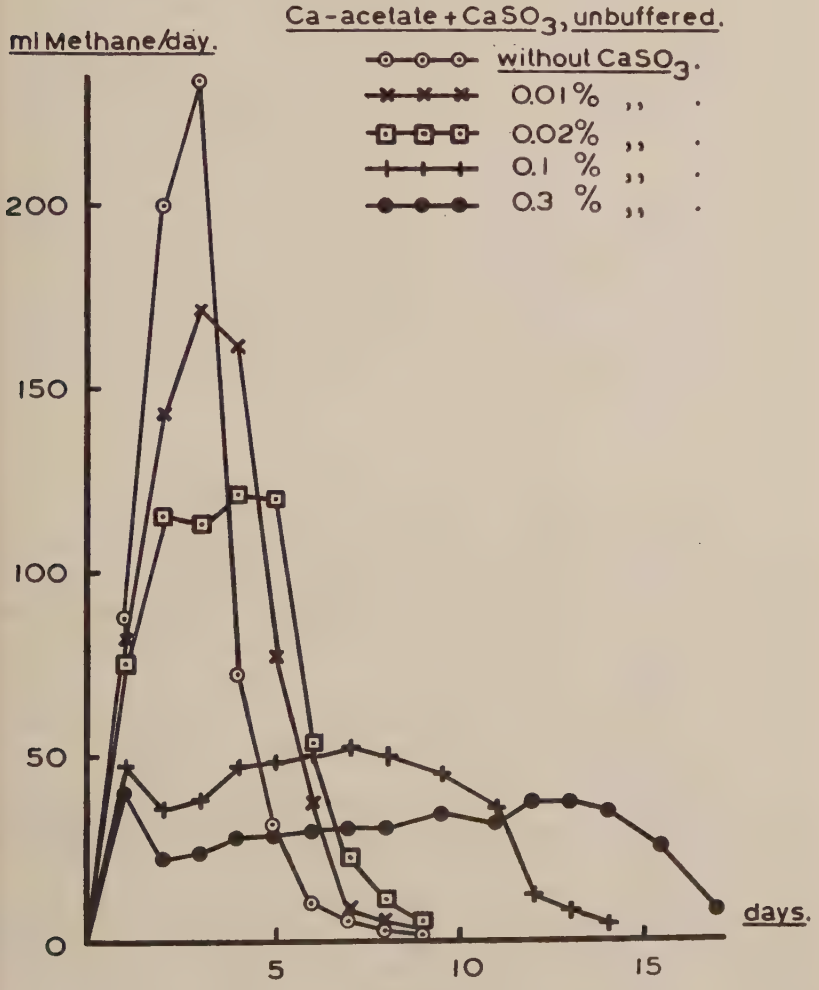

Fig. 11.

closely the composition of the waste liquor from straw-board mills which apply a digestion by lime side by side with sulphite. We may conclude from the above that methane fermentation in this

waste liquor will not be inhibited completely, but nevertheless will be seriously affected by the presence of Na_2SO_3.

§ 8. Influence of calcium sulphite on methane fermentation of calcium acetate.

Finally, the action of solid calcium sulphite on methane fermentation in a medium containing calcium acetate was investigated. The inhibition proved to be practically the same as that obtained with Na_2SO_3 (cf. § 7). In both cases some detrimental effect was already observed upon addition of 0.01 % $CaSO_3$ or Na_2SO_3, respectively, while as much as 0.3 % of either salt did not cause complete inhibition (cf. fig. 11). It appears from figs 10 and 11 that the maximum rate of fermentation in cultures without $CaSO_3$ is 220—235 ml of methane per 24 h. As the culture bottles had a content of amply 250 ml, part of which was taken up by the asbestos this means that one volume of methane can be produced daily from about the same volume of medium containing about 1 % organic material.

§ 9. Discussion.

The results mentioned in § 4 show that inhibition of methane fermentation of sodium acetate by sodium sulphite is greatly dependent on the pH in the range of 6.0—7.5. It is tempting to suggest that this is due to a special sensitivity of the bacteria to HSO_3 ions, as the $[HSO_3'] / [SO_3']$ ratio has the following values in this region [1]):

6.0	6.5	7.0	7.5
0.2	0.063	0.02	0.0063

However, this hypothesis is at variance with the results obtained in fermentations of sodium acetate to which solid calcium sulphite had been added.

In equilibrium with solid $CaSO_3$ a very small concentration of HSO_3' will be maintained in the medium. From the solubility product of $CaSO_3$ at 25° C. (calculated as $5.81.10^{-8}$ from data in the International Critical Tables, Vol. VII) and the second dissociation constant of H_2SO_3 a value for HSO_3' at pH 6.5 of $0.15.10^{-4}$ mol/l is found; in the case of 0.03 % Na_2SO_3 at pH 6.5 this value

[1]) Calculated from the second dissociation constant of H_2SO_3 at 25° C. = 5.10^{-6} (Handbook of Chemistry and Physics 30th Ed. 1948, p. 1426).

is $1.38.10^{-4}$ and for 0.1 % Na_2SO_3 at pH 7.0 it is $1.6 . 10^{-4}$. It appears from figs. 5, 6 and 8, respectively, that in these three cases fermentation was inhibited completely at the end of three days. It may be assumed that the methane bacteria possess an enzyme system responsible for methane production with a very great affinity for HSO_3 ions, this system being blocked by the fixation of HSO_3'. This blocking then would be complete even in a concentration of HSO_3' as low as $0.15.10^{-4}$ mol/l; it is true that the absorption of HSO_3' will tend to lower the concentration in the medium, but then HSO_3' will be formed anew from the excess of undissolved $CaSO_3$ (0.1 %), or of SO_3 ions in the case of Na_2SO_3. The HSO_3' concentration in a 0.03 % Na_2SO_3 solution at pH 6.5 is about nine times that of a saturated $CaSO_3$ solution at that pH, but the supply of SO_3'' in the former case is about $^1/_3$ of that of $CaSO_3$ in the latter. However, in a 0.1 % solution of Na_2SO_3 at pH 7.0 the HSO_3' concentration and the inhibitive power are about the same as in a 0.03 % solution at pH 6.5, whereas the SO_3'' supply in the former solution is about thrice that in the latter (a 0.05 % Na_2SO_3 solution caused at pH 7.0 only slight inhibition although it contains $0.78.10^{-4}$ mol/l HSO_3').

These facts show that the HSO_3' concentration cannot be the only factor governing the inhibition of methane fermentation by sulphite. Perhaps the affinity of the enzyme system for HSO_3' strongly decreases with increasing pH.

The results obtained with the combinations calcium acetate-sodium sulphite and calcium acetate-calcium sulphite are still more difficult to explain. It may be assumed that the pH in these cultures was about 6.5. As it is not known to what extent sulphite was precipated in the former case, it is difficult to calculate the HSO_3' concentration. Assuming, however, complete precipitation of sulphite as $CaSO_3$, we find for a pH of 6.5 and an initial concentration of 0.15 % Na_2SO_3, an SO_3'' concentration of $1.14.10^{-6}$ and an HSO_3' concentration of $7.2.10^{-8}$ mol/l. In the latter case (1 % Ca-acetate and 0.1 % $CaSO_3$) the initial concentrations of SO_3'' and HSO_3' cannot have been higher than these values, as no more sulphite can pass into solution than the amount corresponding to the solubility product of $CaSO_3$ and the ratio $[HSO_3'] / [SO_3'']$ at pH 6.5. It is unlikely that there is so large an affinity of the enzyme system of the bacteria for HSO_3' that an equilibrium concentration of $7.2.10^{-8}$ mol/l will suffice to saturate the enzyme,

in the presence of an adequate supply of solid $CaSO_3$. Even if it is assumed that the affinity for SO_3'' is as great as that for HSO_3'' the saturating concentration of the poison seems incredibly low.

Therefore, further investigations will be necessary to elucidate the mechanism of sulphite poisoning of methane fermentation.

§ 10. Concluding Remarks.

The composition of the waste liquor of a straw-board mill applying exclusively lime digestion was found to be as follows. The total dry matter in the waste amounted to 1.69 %. This was composed of:

ash	37.3 %
protein (N × 6.25)	4.0 %
reducing sugars	2.0 %
acetic acid [1])	9.8 %
lignin	7.1 %
pentosan	11.5 %
remainder (probably mainly hexosan)	28.3 %

This analysis shows that almost $^2/_3$ of the dry matter is organic substances. About 10 % of the dry matter is acetic acid. As the greater part of the ash consists of lime, acetic acid will be present in the form of calcium acetate. Since calcium acetate is an exceptionally favourable substrate for methane bacteria, it will be one of the first constituents to be converted to methane and carbon dioxide. Therefore, in the methane fermentation of this waste the initial phase will be essentially a fermentation of calcium acetate.

Mills which use both lime and Na_2SO_3 in pulping will discharge in their waste liquor some Na_2SO_3. As the pH of the fermenting liquor is about 6.5 a concentration as low as 0.01 % Na_2SO_3 may have an inhibitory effect. In an experiment with increasing quantities of sulphite waste liquor added to cultures with sodium acetate the inhibition of methane production was found to be in agreement with the amount of sulphite [2]) present in this liquor. This proves that the detrimental action of sulphite waste liquor is actually due to its sulphite content. Therefore, there is need of a method to counteract this detrimental action of sulphites.

In theory this may be accomplished in two ways. First, it might be tried to oxidize the sulphite to sulphate by means of an air

[1]) Present in the form of acetate.

[2]) Determined by iodine titration.

current bubbling through the liquor. Preliminary experiments in this direction were not encouraging, however.

Therefore, it seems a better proposition to subject the sulphite to a microbiological reduction by *Vibrio Rübentschickii*, which effects hydrogenation of both sulphates and sulphites to hydrogen sulphide with fatty acids (e.g. acetic acid) as a hydrogen donator (Baars(1)). Preliminary experiments with regard to this possibility yielded promising results and investigations will be continued in this direction.

Summary.

It has been proved that methane fermentation of sodium and calcium acetates is strongly inhibited by both sodium and calcium sulphites. In the region of optimum pH of this fermentation (pH 6.0 to 6.5) a concentration of 0.003 to 0.01 % sodium sulphite is enough to produce perceptible inhibition. At a concentration of 0.1 % Na_2SO_3 methane fermentation was inhibited completely for some days. Hereafter a recovery took place, most probably due to the development of micro-organisms converting sulphites to H_2S. At a pH higher than 6.5 methane fermentation was less sensitive to Na_2SO_3 and $CaSO_3$.

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ISOLATION OF ORNITHOSIS VIRUS FROM PIGEONS IN THE NETHERLANDS

by

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Introduction.

Complement fixation tests, performed by F. Dekking, pointed out that psittacosis (ornithosis) must be wide spread among pigeons in Holland, particularly among homing pigeons. For the first time in our country we were able to isolate an ornithosis like virus from pigeons, kindly provided by H. J. Stol.

In former days the psittacosis virus has only been isolated from parrotlike birds like amazon parrots, parrakeets and other psittacidae and as a rule only small family epidemics occurred following inhalation of whirled dried feces from the birds, kept as household animals, which may be ill, or, although healthy, may be virus-carriers, excreting the virus with the feces. In birds the virus rests particularly in the spleen, liver and kidneys, the lungs being seldom attacked in contrast to man in which the disease predominates as an atypical pneumonia with viremia.

It became evident that not only psittacidae in wild state as well as kept as pets, in the whole continent of America, parts of Europe and Australia, acted as virus carriers and spreaders, but that numerous kinds of other birds in bird trades and breeding cages and those in a wild state were infected with the virus. Meyer (8, 9) differentiates the virus, isolated from psittacidae and named by him „Psittacosis virus", from the similar virus isolated from other birds. He has based the difference in name only on the source from which the virus has been isolated, although minor other differences may be found as a rule. To our mind making a difference like this may not be in the interest of public health, for in the Netherlands

the dutch official regulations concerning the control of communicable diseases only deal with „psittacosis".

In 1940 PINKERTON and SWANK (10) in the U.S., isolated from young pigeons, kept on a diet deficient in thiamine, a virus with the characteristics of psittacosis virus with the exception that an intraperitoneal inoculation of mice with this virus did not provoke any sign of disease, in contrast to the classical psittacosis virus. The next year, MEYER (8, 9) isolated from the sputum of a pigeon-fancier, suffering from an atypical pneumonia a psittacosis-like virus; 19 out of 33 pigeons from the patients loft showed a positive complement fixation test with psittacosis antigen and from 4 pigeons a virus strain could be isolated, quite identical with the strain isolated from the patient.

COLES (4), in South Africa, in the same year, demonstrated the presence of a psittacosis like virus in pigeons and during the 2nd world war, ANDREWES and MILLS (1) and HUGHES (7) also succeeded in it.

The psittacosis or ornithosis virus causes an infection of the nestlings; the survivors remain virus carrier and may, as adult birds, in their turn infect their progeny. In nestlings and young pigeons a pericarditis and fibrinous peritonitis may be found. An enlarged spleen is rather common in the disease (as well as in the carrier state) and may lead to a lethal intraperitoneal hemorrhage (HUGHES). Pinheadlike necrotic foci may be demonstrated in the liver (HUGHES). The clinical symptoms in pigeons are not characteristic. Emaciation and diarrhoea (which may be a complicating salmonellosis) may occur as well as a sudden death from the healthy state. HUGHES also observed in some cases opisthotonus.

METHODS AND MATERIALS.

H. J. STOL, vet. surgeon, provided us kindly with 4 pigeons, in which we made an attempt to demonstrate ornithosis virus. F. DEKKING kindly carried out complement fixation tests in pigeons as well as in laboratory animals infected with the virus that we could isolate from 2 pigeons.

In our attemps to isolate an ornithosis like virus we only made use of mice although the embryonated hen's egg is recommended by many authors: BURNET and ROUNTREE (2), YANAMURA and MEYER (13), BURNET and FOLEY (3) and WILLIAMS (11). The

canary and Javanese rice birds are stated too as being very susceptible to the virus.

We were aware of the fact, that spontaneous infection with a psittacosis (ornithosis) like virus may occur in mice (bronchopneumonia virus) but we could eliminate it on reasonable grounds.

Pigeon Nr. 1 showed only symptoms of a vit. B. deficiency and as PINKERTON and SWANK (10) could isolate a psittacosis like virus from pigeons, kept on a diet deficient in thiamin (vit. B_1), the bloodserum was examined on the presence of complement fixing antibodies to psittacosis-antigen. The titer was 1 : 320++. Of course, in this and the other cases a complete history was made, including anamnesis, state of health, examination especially for salmonellosis (bloodserum, feces), intestinal parasites and other common diseases of pigeons and an obduction protocol. We made a suspension of liver, spleen and kidneys and, after bacteriological sterility tests, some mice have been inoculated by intracerebral and by intra abdominal route, but we did not succeed in provoking any signs of disease; in 3 mice, killed one month after inoculation the complement fixation test was negative.

Ten pigeons from the same fancier were examined by F. DEKKING on the presence of complement fixing antibodies to psittacosis virus with this result: 2 birds negative, 7 had a titer 1 : 80++++ and 1 had a titer 1 : 80+. We tried to isolate the virus from another pigeon of this flock with a negative complement fixing test, by means of intracerebral and intraperitoneal inoculation of mice and intranasal instillation of an suspension of the organs of the pigeon in hamsters, but our attemps were unsuccesful.

We had more luck with 2 not quite healthy pigeons provided by another pigeon fancier. We could exclude salmonellosis and intestinal parasites. The sera of the two birds showed no complement fixing antibodies to psittacosis antigen. At obduction we observed only in one of the two birds a somewhat enlarged spleen and hyperemia of the intestines. By intranasal instillation of hamsters with a combined suspension of the organs (spleen, liver, kidneys) and intranasal and intraperitoneal infection of mice we failed to demonstrate any pathogenic agent but by intracerebral route we succeeded to provoke illness and death in mice. In the first passage, death occurred within 7 days but in the second and further passages deaths occurred regularly within 4 days. Mice, surviving the infection and killed after 1—2 months, frequently showed an enlarged

spleen and a positive complement fixation test to psittacosis antigen. We thought to be dealing with an infectious agent of the psittacosis-ornithosis-lymphogranuloma venereum group and based our opinion on these and continued experiments:

1. In touch preparations, made from different organs and stained by Castaneda's method with ,,blue Borrèl'' (a suggestion of F. DEKKING), we observed intra and extracellular masses of spherical or coccoid particles, different in size, stained blue in contrast to the reddish-brown colour of the nuclei and cytoplasma of the cells.
2. A positive complement fixation test in mice which survived the experimental infection, e.g., mouse of the 2nd passage, inoculated intracerebrally and intraperitoneally and killed after a month, titer $1 : 60\pm$.
3. Bacteriological sterility (aerobic and anaerobic control) of the organ-suspensions.
4. The source: 2 pigeons. Toxoplasmosis could be excluded.
5. Latent or active virus infections in our stock mice, particulary virus bronchopneumonia, could be excluded.
6. The viral agent proved to be very active by intracerebral and intranasal inoculation of mice (a heavy consolidation of the lungs was observed after intranasal infection) causing deaths in 4 days; by intraperitoneal inoculation the agent proved to be less active, although deaths could be observed in 4—8 days.
7. Young pigeons, inoculated intracerebrally with an organ suspension of infected mice, died within 4—8 days and inoculation of brain suspension provoked an experimental disease in mice which, based on the preceding numbers, was regarded to be ornithosis.
8. Persons regularly working with viruses are exposed to contamination and especially in the classical psittacosis the possibility of a latent or perhaps an active infection really exists; in 4 of those persons, who have been working with our virus strain, the complement fixation test with psittacosis antigen proved to be negative. In our opinion it might be concluded from this fact that our virus is rather a mitigated strain of psittacosis virus as the ornithosis virus may be.
9. The complement fixation test in psittacosis-ornithosis-lymphogranuloma venereum is a group reaction; an antigen, prepared from the lungs of intranasally inoculated mice, showed a com-

plement fixation titer of 1 : 16 with a known positive lymphogranuloma venereum serum.

According to the method applied by HILLEMAN (6), we injected two chickens serially with the living virus in order to obtain neutralizing and/or complement fixing antibodies, but we failed to provoke them.

Three ferrets were inoculated intranasally, intracerebrally and intraperitoneally respectively with the virus but the animals remained healthy; only the intracerebrally inoculated ferret showed some rises of temperature during the first week. Serum, taken from the 3 animals 16 days after the exposure to the virus, proved to be anticomplementary. The intranasally infected ferret was killed and showed no lung lesions.

Three hamsters were infected in the same way, the intranasally infected animal died on the 9th day, but showed only a few minimal lung lesions in which we could detect some virus colonies with difficulty. The same was true with the intracerebrally inoculated hamster that also died on the 9th day. The intra-abdominally inoculated hamster remained healthy.

Summary.

Psittacosis (ornithosis) virus has been isolated from Dutch pigeons for the first time. Some characteristics of the virus strain could be determined in experiments with animals.

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SURVEY OF VARIOUS CULTURE MEDIA FOR THE ISOLATION OF SALMONELLA'S AND SHIGELLA'S.

by

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(Received May 7, 1949).

In most of the routine laboratories the earliest simple media for the isolation of pathogenic enteric microbes (Endo, Conradi-Drigalski) have now been abandoned and are replaced by more selective ones, often combined with culture in fluid enrichment media.

In previous communications (6, 7) one of us (R) pointed out, that the enrichment media of MULLER (tetrathionate-broth) and KAUFFMANN's modification (addition of bile and brilliantgreen) have again been surpassed by newer ones (LEIFSON's selenite medium (4) and brilliantgreen-picric-citric acid-broth (RUYS)). From the enrichment media we subcultured on Endo's medium because we were short of chemicals for the selective ones. However, it may be preferable to use for this purpose the S.S. plate (see below).

WILSON and BLAIR's plate was the first highly selective solid medium, especially for the isolation of *S. typhi*. A drawback is that not only the growth of most of the non-pathogens but also that of the Shigella's is suppressed and to some extent also that of several Salmonella's. *S. typhi* suffers the least from the adverse conditions in this medium and acquires thereby the advantage over other microbes. This medium was the first one by which the isolation of *S. typhi* and *S. schottmülleri* from contaminated surface water succeeded with some regularity (5).

WILSON and BLAIR's medium is difficult to prepare. It does not always give constant results and it deteriorates rapidly. HAJNA (2) tried to improve it by replacing bismuth-ammonium-citrate by

bismuth-citrate. This new medium is even more selective and is less liable to cause unexpected difficulties.

Comparison of these media with the desoxycholate-citrate medium of LEIFSON, however, showed that several positive cases, especially of *S. typhi* infections, were missed by the HAJNA and WILSON-BLAIR plates. Besides, the desoxycholate plate is an excellent medium for the isolation of *Sh. paradysenteriae* and a little less for *Sh. sonnei*.

In the Salmonella-Shigella (S.S.) medium of Difco (1) containing bilesalts, sodium citrate, thiosulfate and ferric citrate, an attempt is made to combine the good qualities of several. It is less selective than the more inhibitive media like HAJNA-, WILSON-BLAIR- and desoxycholate-citrate plates. Therefore there may be some advantage in using it in addition to the other ones. It has a wide range of possibilities because it allows the growth of all the indigenous enteric pathogens, be it is not always optimally. *Proteus* does not swarm on it.

For the isolation of Salmonella's KAUFFMANN's (7) brilliantgreen agar plate is also widely used. However, all the Shigella's are inhibited and only very few *S. typhi* will grow on it, so its usefulness is restricted to the isolation of *S. schottmülleri* and perhaps some of the other Salmonella's.

In order to find out which combination of media would yield optimum results with the least amount of work we made several comparisons, the results of which will be shown below.

Micro-organism	number pos. specimens	media tried				
		WILSON-BLAIR plate	selenite broth	desoxy-cholate-citrate plate	S.S. plate	brilliant green ESBACH broth
S. paratyphi A	4	1	—	1	—	4
S. typhi	139	83	112	122	81	—
	88	48	68	63	—	—
S. schottmülleri	187	—	167	26	81	129
S. typhi-murium	48 / 31 = 79	—	33 / 28 = 61	26 / 14 = 40	15 / —	26 / 24 = 50
S. enteritidis	11	—	2	7	2	4
S. bareilly	3	—	1	0	0	3
Sh. sonnei	127	—	93	40	80	—
Sh. paradysenteriae	94	—	—	87	47	—
Sh. newcastle (Boyd 88)	7	—	1	4	5	—

S. typhi. The best results are obtained with the desoxycholate-citrate medium and after enrichment in selenite broth. In a series of 63 positive stools WILSON and BLAIR's plates were compared with HAJNA's. The former yielded 42 positives the latter 56. We therefore think that for *S. typhi* the best combination is now the desoxycholate-citrate and HAJNA's plates, and the enrichment selenite medium.

S. schottmülleri. Primary culture on solid media gave, especially on desoxycholate-citrate agar, poor results. In a later series we tried KAUFFMANN's brilliantgreen-agar plates. Of a hundred cases positive after enrichment in selenite broth only 62 were positive on the KAUFFMANN's plates. However, this one may replace the desoxycholate-citrate plate, but it is not as good as it is often judged. Enrichment in brilliantgreen-ESBACH broth stands behind that in selenite but still gives fairly good results.

S. typhi-murium. The number of positive specimens is not large but the results suggest that the value of the solid media tested is the reverse of that for *S. schottmülleri*, the desoxycholate-citrate plate now being superior to the S.S. medium. The fluid media show the same relation as with *S. schottmülleri*. Plating on KAUFFMANN's brilliantgreen-agar stood behind enrichment in selenite broth, the former giving only 11 positives out of 22 with the latter.

The results with less frequent Salmonella's are also shown in the table, but it is difficult to draw conclusion from such small numbers. Brilliantgreen-ESBACH broth seems to yield fairly good results. We were astonished by the relative large number of *S. enteritidis* cultures on desoxycholate-citrate plates. Of all the salmonelloses it is only in infections with *S. typhi* that this medium is superior to the other ones tested.

Sh. sonnei. The S.S. plate gives more positive results than the desoxycholate-citrate plate, but enrichment in selenite broth still increases the number of positive findings.

Sh. paradysenteriae. The picture on the various media is the reverse of that obtained with *Sh. sonnei.* Desoxycholate-citrate plates give better results than the S.S. plates. Selenite broth is no good at all; only 2 out of 54 positive specimens which were inoculated also into selenite broth yielded colonies of *Sh. paradysenteriae* on subculture.

Sh. newcastle. The number of examinations is far too small to allow of our drawing any other conclusion than that the selenite

broth seems to offer little advantage and that the desoxycholate-citrate and S.S.plates probably give fairly good results. During the war when only desoxycholate-citrate and Endo plates were available, we found the former quite satisfactory, yielding 197 positive cultures out of 200 positive specimens against Endo's medium only 88. However, a combination with S.S.plates may improve the score.

Discussion.

From the facts related above it follows that even closely related species of enteric pathogens behaved quite differently in the seven media tested. The larger the number of media the higher the possibility of obtaining optimum results, not only because the quantity of material examined is larger but also because of the chance to include in the series also that medium which favours mostly the growth of the special pathogen. In the search for Salmonella's and Shigella's the stools should always be cultured on several media at least on two. If the causative agent is unknown and if one has to limit oneself to two media, the desoxycholate-citrate plate and the enrichment selenite medium should be chosen. In a typhoidlike disease HAJNA's medium may be added for *S. typhi*, KAUFFMANN's plate and the brilliantgreen-ESBACH broth for *S. schottmülleri*. In cases with gastro-enteritis an S.S.plate may be added for Shigella's but for Salmonella's brilliantgreen-ESBACH broth and KAUFFMANN's plate seem to be preferable.

Summary.

The different behaviour of related intestinal pathogens on various media for isolation is stressed.

For the indigenous Salmonella's and Shigella's a certain combination of selective and enrichment media is recommended. The formula of these media are as follows:

1. LEIFSON, desoxycholate-citrate medium (J. Path. a. Bact. **40**, 581, 1935).

Pork (meat) infusion	1000 ml
peptone (Bacto, Fairchild)	10 g
agar-agar	20 g
lactose	10 g
sodium citrate (2 $Na_3C_6H_5O_7$. 11 H_2O)	25 g

sodium-desoxycholate	5 g
ferric ammonium citrate (green scales)	2 g
neutral red (1 : 50.000) pH 7.4	20 mg

2. LEIFSON, modified selenite medium. (Am. J. Hyg. **24**, 423, 1936).

sodium biselenite	0.4 g
mannitol	0.4 g
peptone (Bacto)	0.5 g
sodium di phosphate	1 g
distilled water	100 ml

pH = 7.

3. HAJNA's modification of the WILSON-BLAIR plate (J. Lab. Clin. Med. **23**, 1185, 1938).

a.	agar-agar	30 g
	beef-extract	5 g
	peptone (Bacto)	10 g
	dextrose	5 g
	water	1000 g
b.	water	400 ml
	ammonium hydroxyde (30 %)	12 ml
	sodium sulphide	80 g
	bismuth citrate	24 g
	sodium di phosphate	42 g

Add to *b* 40 ml of a solution of 4 g $FeSO_4$ (7 H_2O) in 50 ml water. Take 1000 ml of the agar base (*a*), 70 ml of the solution (*b*) and add 4 ml brilliant-green solution (10 %). Final pH = 7.8.

4. Salmonella-Shigella medium. Difco Manual. 1948, p. 97.

Beef-extract	5 g
proteose peptone	5 g
lactose	10 g
bile salts, Na. tauroglycholate (Bacto)	8.5 g
sodium citrate	8.5 g
sodium thiosulfate	8.5 g
ferric citrate	1 g
agar-agar	13.5 g
brilliantgreen	0.33 g
neutral red	0.025 g
distilled water	1000 ml

Final pH = 7.

5. Brilliantgreen-Esbach enrichment medium (Ruys, Zentralbl. f. Bakt. I, **132**, 349, 1934).

nutrient broth (pH 7.2)	1000 ml
brilliantgreen solution (1 : 1000)	10 ml
Esbach's reagent (1 g picric acid, 2 g citric acid in 100 ml water)	20 ml

6. F. Kauffmann, Zeitschr. f. Hyg. u. Inf. Krankh. **119**, 233, 1937.

distilled water	100 ml
beef-extract	0.5 g
peptone	1 g
sodium chloride	0.5 g
lactose	1.5 g
phenolred solution (40 ml 0.1 n NaOH + 460 ml water + 1 g phenolred)	4 ml
brilliantgreen solution (1 : 1000)	0.5 ml
agar-agar	2.5 g

Final pH = 7.2.

References.

1. Difco Manual. 1948, p. 97. – 2. A. A. Hajna and C. A. Perry, J. Lab. Clin. Med. **23**, 1185, 1938. – 3. F. Kauffmann, Zeitschr. f. Hyg. u. Inf. Krankh. **119**, 233, 1937. – 4. E. Leifson, Am. J. Hyg. **24**, 423, 1936. – 5. A. Ch. Ruys, Proc. Kon. Akad. v. Wet. **39**, 780, 1936. – 6. A. Ch. Ruys, Brit: Med. J. **1**, 606, 1940. – 7. A. Ch. Ruys, Am. J. Public Health **38**, 1219, 1948.

(Aus dem Instituut Pasteur, Bandoeng, Java).

HAEMAGGLUTINATION DURCH POCKENVIRUS

6. Ueber Inhibine aus normaler Lunge für die Vaccinevirus-Haemagglutination [1])

von

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(Eingegangen am 5 April 1949).

Es ist bekannt, dass die Haemagglutination einzelner Virusarten durch die verschiedensten Stoffe gehemmt werden kann. STONE and BURNET (15) fanden ein derartiges Inhibin in roher Kalbslymphe und sprachen ihm die Natur eines Antistoffes zu, ähnlich den Serumantistoffen. Eine derartige Inhibinwirkung durch Extrakte aus normalen Organen wurde auch in diesem Institut bei zahlreichen Versuchen über die Haemagglutination durch Karbouwen-Vaccine gefunden. Am regelmässigsten waren die Hemmungswirkungen bei Lungenextrakten. Da für die Pockeninfektion die porte d'entrée in den Atmungsorganen zu suchen ist, schien eine genauere Untersuchung der in der Lunge nachweisbaren Hemmungsstoffe gerechtfertigt. Von einem Inhibin muss man eine deutliche Affinität zu dem betreffenden Virus annehmen, die mindestens ebenso gross ist wie die Affinität der benutzten Erythrocyten.

M e t h o d i k.

Benutzt wurde Vaccinevirus vom Karbouw, das 3 Tage nach cutaner Impfung geerntet war. Die Aufbewahrung erfolgte bei —15° C. Die sehr fein verriebene 10%ige Suspension wurde ca. 10 Minuten centrifugiert, und die überstehende Flüssigkeit verwendet.

Das Blut stammt von javanischen Landhühner, wurde 3 mal gewaschen und meist frisch benutzt, nur gelegentlich nach 24 Stunden. Es wurden nur Hühner gebraucht, die eine sehr hohe Empfindlichkeit aufwiesen.

[1]) Mitteilung 5: Antonie van Leeuwenhoek **15**, 53, 1949.

Die Lungen stammten von den verschiedensten Tieren, auch kamen einige Menschenlungen zur Untersuchung, welche von durchaus gesunden Personen stammten, die durch Unfall ums Leben gekommen waren. Die Versuche wurden sowohl während der trocknen Zeit (± 22° C.) als auch während der Regenzeit (± 18° C.) durchgeführt, wobei sich keine erkennbaren Unterschiede zeigten.

Die Bindung zwischen Inhibin aus den Lungen und Vaccinevirus erfolgte 30 Minuten lang bei 37° C. Nach Blutzufügung blieben die Röhrchen bei Zimmertemperatur stehen, und die Ablesung konnte in der Regel nach ca. 1½ Stunden erfolgen.

Antisera gegen Inhibine wurden vom Kaninchen gewonnen. Benutzt wurden die schwachen javanischen Landkaninchen von ca. 1 Kilo Gewicht. Die Immunisierung erfolgte intravenös mit 10%iger Lungen-Suspension, die ca. 10 Minuten bei 2000 Umdrehungen zentrifugiert worden war. Von der überstehenden Flüssigkeit wurde je 0,5 oder 1,0 ccm an 6 aufeinander folgenden Tagen intravenös injiziert. Die Blutentnahme erfolgte 8—10 Tage nach der letzten Injektion. Kaninchenlunge erwies sich als sehr toxisch, Mäuselunge wurde dagegen gut vertragen. Die Sera wurden im inaktiven Zustande untersucht. Es wurden nur Kaninchen benutzt, die vor der Immunisierung keine hemmenden Serumstoffe für die Vaccinevirus-Haemagglutination aufwiesen und die ebensowenig die Inhibinwirkung von Lungenextrakten hemmten.

Experimenteller Teil.

Virusadsorption durch Lungenbrei und Hemmung durch Lungenextrakt.

Von 5 bunten Ratten wurde je 1 g Lunge, Milz, Leber und Gehirn im Mörser sehr fein verrieben, in 2 ccm physiologischer Kochzalzlösung suspendiert und bei 2000 Umdrehungen centrifugiert. Die überstehende Flüssigkeit wurde zum Hemmungsversuch benutzt: Fallende Verdünnungen hiervon wurden in der Menge von 0,2 ccm mit 0,2 ccm der doppelten agglutinierenden Dosis Vaccine gemengt und 30 Minuten bei 37° C. gehalten. Die Ergebnisse finden sich in Tabelle 1 zusammengestellt.

Der Niederschlag wurde noch einmal mit je 10 ccm physiologischer Kochsalzlösung gewaschen und hiernach met 3 ccm Vaccinevirus 1/10 gemischt und 1 Stunde lang bei 37° C. gehalten. Hierauf

Tabelle 1.

Hemmung der Vaccinevirus-Haemagglutination durch Organextrakte.

Ratte No.	Die doppelte agglutinierende Virusdosis wird durch Organextrakte gehemmt bis zur Verdünnung von:			
	Lunge	Milz	Leber	Gehirn
11	1/144	1/9 nicht	1/9 nicht	1/9 nicht
12	1/72	1/9 nicht	1/9 nicht	1/9 nicht
13	1/144	1/9 nicht	1/9 nicht	1/9 nicht
14	1/144	1/9 nicht	1/9 nicht	1/9 nicht
15	1/144	1/9 nicht	1/9 nicht	1/9 nicht

wurde die abzentrifugierte Virussuspension in fallenden Verdünnungen zu je 0,25 ccm mit Hühnerblutsuspension von 1 % gemengt, um den Endtiter der Haemagglutination zu bestimmen. Als Kontrolle wurde das unbehandelte 10%ige Virus gleichfalls austitriert. Die Ergebnisse finden sich in der Tabelle 2 zusammengestellt.

Tabelle 2.

Adsorption von Vaccinevirus an gewaschene Organzellen.

Ratte No.	Endtiter der Virus-Haemagglutination nach Behandlung mit gewaschenem Organbrei:				Unbehandeltes Virus (Kontrolle)
	Lunge	Milz	Leber	Gehirn	
11	1/25 neg.	1/200	1/400	1/400	1/800
12	1/25 neg.	1/400	1/800	1/800	
13	1/25 neg.	1/800	1/800	1/800	
14	1/25 neg.	1/400	1/100	1/800	
15	1/25 neg.	1/400	1/800	1/400	

Aus der Tabelle 1 geht hervor, dass die Extrakte aus den Rattenlungen in jedem Fall die doppelte agglutinierende Virusdosis deutlich hemmten und zwar in 4 Fällen bis zur Endverdünnung von 1/144 und in einem Fall bis zur Endverdünnung von 1/72. Demgegenüber hemmten die verschiedenen Extrakte aus Milz, Leber und Gehirn in der Verdünnung von 1/9 in keinem Fall. Die Lungen von allen 5 Tieren zeigen also sehr deutliche Inhibinwirkung.

Die Fähigkeit der gewaschenen Organzellen, Vaccinevirus zu adsorbieren, geht aus der Tabelle 2 hervor. In der Kontrollereihe agglutinierte das unbehandelte Virus noch bis zur Verdünnung von 1/800. Demgegenüber zeigte das Virus nach Adsorption mit Rattenlungenbrei in keinem Fall Agglutination, selbst nicht in den stärksten untersuchten Konzentrationen von 1/25. Es hatte also eine starke Adsorption des Virus an die Lungenzellen stattgefunden. Bei den Adsorptionsversuchen mit anderen Organen hatte in diesem Versuch allerdings auch einmal die Milz den ursprünglichen Virus Titer auf 1/4 gesenkt, und einmal die Leber auf 1/8.

Die Rattenlunge dürfte also im Vergleich mit den anderen Organen wohl die meisten virusaffinen Rezeptoren aufweisen. Diese sind nicht nur zellständig, sondern auch imstande, in den Gewebsextrakt überzugehen.

Bei 3 Kaninchen erwiesen sich zwar die Lungenextrakte als stark hemmend, aber Extrakte aus Leber, Milz, Niere, Herz und Gehirn waren wirkungslos.

Etwas anders verhielten sich Meerschweinchen, da hier auch in anderen Organen nicht selten Inhibine nachgewiesen werden konnten. So zeigten beispielsweise von 12 untersuchten Tieren Inhibinwirkung gegen die doppelte agglutinierende Virusdosis:

10 Tiere im Extrakt der Lungen
6 Tiere im Extrakt der Nieren
5 Tiere im Extrakt der Milz
kein Tier im Extrakt der Leber.

Bei allen diesen 12 Meerschweinchen erwies sich das Blut sowohl im aktiven als auch im inaktiven Zustande als vollkommen frei von hemmenden Stoffen.

U.a. wurden auch zwei Affen (*Macaca irus mordax*) untersucht, die nach cerebraler Infektion mit Newcastle disease-Virus im moribunden Zustand getötet waren. Eines der Tiere wies in Lunge und Niere reichlich Inhibine auf, in Leber und Herz wenig und in der Milz gar nicht. Der andere Affe hatte nur in Niere und Leber Inhibine, nicht dagegen in Lunge, Milz, Herzmuskel, Pancreas und Skelettmuskulatur. Das Serum des ersten Affen wirkte im aktiven Zustande bis zur Verdünnung von 1/20, das des anderen bis zu 1/40 hemmend. Inaktiv waren beide Seren wirkungslos.

In den Organextrakten von zahlreichen Meerschweinchen und Ratten, die gegen Vaccinevirus immunisiert waren, fanden sich

sehr häufig noch in relativ hohen Verdünnungen hemmende Stoffe, die aber fast stets einigermassen mit den entsprechenden Anti-Haemagglutininen des Serums an Stärke übereinstimmten und wohl mit diesen zu identifizieren waren.

Wirksamkeit verschiedener Lungenextrakte.

Nicht bei allen untersuchten Tierarten fand sich die Wirkung der Lungenextrakte gleichmässig ausgebildet.

Wirksam erwiesen sich stets in hohen Verdünnungen die Extrakte aus Rinderlungen, die vom Schlachthaus bezogen wurden. Ferner waren alle 7 untersuchten Menschenlungen deutlich aktiv. Von 10 Mäusen zeigten 2 Inhibinwirkung auf die doppelte agglutinierende Virusdosis in der Verdünnung von 1/400, 6 in der Verdünnung von 1/200 und 2 in der Verdünnung von 1/100

Tabelle 3.

Verschiedenheit der hemmenden Wirkung von Lungenextrakt.

Kaninchen No.	Multiplum der agglutinierenden Dosis	Verdünnung der Lungenextraktes				
		1/30	1/60	1/120	1/240	1/480
7	1×	0	0	0	0	0
	2×	+	+	+	+	+
	4×	+	+	+	+	+
	8×	+	+	+	+	+
8	1×	0	0	0	0	0
	2×	0	0	0	+	+
	4×	0	0	0	+	+
	8×	0	+	+	+	+

Inhibinwirkung zeigten endlich auch alle untersuchten Kaninchenlungen bis auf eine. In der Tabelle 3 ist die Inhibinwirkung von 2 Kaninchenlungen nebeneinander gestellt und zwar gegen die einfache bis achtfache Konzentration der agglutinierenden Dosis. Es zeigt sich ein deutlicher Unterschied in der Stärke, denn während der Extrakt von Kaninchen 7 nur gegen die einfache agglutinierende Virusdosis wirksam ist, hemmt der Extrakt von Kaninchen 8 auch noch die achtfache Viruskonzentration, wenn auch nur in der ersten Verdünnung. Zwischen diesen beiden Ex-

tremen fanden sich bei anderen Tieren alle möglichen Zwischenstufen.

Die Lunge eines jungen Hundes enthielt viel Inhibin, die Lunge eines Reisvogels (*Paddà oryzivorus*) sehr wenig.

Nicht regelmässig fanden sich Inhibine in den Lungen von schwarz-weissen Ratten und Meerschweinchen. Bei einer Meerschweinchenserie waren beispielweise nur 10 von 12 Tieren positiv, in einer anderen 16 von 20. Es handelte sich hierbei stets um Tiere, die mit Diphtherie- oder Tetanustoxin vorbehandelt waren. Bei den Ratten waren mitunter alle Tiere positiv, gelegentlich aber fanden sich verschiedene Tiere ohne Inhibine in den Lungen. So hemmten beispielweise die Lungenextrakte von 9 Tieren einer Serie in 4 Fällen bis 1/400 (deren Serum einmal bei 1/20 und dreimal nicht bei 1/5), in 2 Fällen bis 1/200 (deren Serum einmal bei 1/20 und einmal nicht bei 1/5), und in 3 Fällen nicht in der Verdünnung 1/25 (deren Serum einmal bei 1/10 und zweimal nicht bei 1/5) die doppelte agglutinierende Virusdosis.

Thermolabilität der Lungenextrakte.

Wichtig erschien die Feststellung der Thermolabilität der Inhibine in den Lungenextrakten. Es zeigte sich, dass es keineswegs gleichgültig war, in welcher Konzentration die Extrakte erhitzt wurden. In Konzentrationen von beispielsweise 1 : 4 wurde bei höheren Temperaturen sehr viel Eiweiss ausgeflockt und riss anscheinend dabei wirksame Stoffe mit sodass infolgedessen konzentrierte Extrakte schneller inaktiviert wurden als verdünntere. Als brauchbar erwies sich eine Lungensuspension von 1/8—1/10.

Tabelle 4.

Thermolabilität von Rinderlunge: Hemmung der Haemagglutination.

Erwärmung auf:	Hemmung der Agglutination nach Erwärmung von					
	0	10′	20′	30′	1h	2h
nativ:	1/200	—	—	—	—	—
45° C.		1/800	1/800	1/400	1/200	1/400
50° C.		1/400	1/400	1/800	1/100	1/200
55° C.		1/100	1/50	1/25 nicht	1/25 nicht	1/25 nicht
65° C.		1/200	1/50	1/25 nicht	1/25 nicht	
70° C.		/1200	1/200	1/25 nicht	1/25 nicht	
80° C.		1/25 nicht	1/25 nicht	1/25 nicht	1/25 nicht	

In Tabelle 4 findet sich eine Versuchsserie wiedergegeben, in welcher Rinderlungenextrakt in der Verdünnung 1/8,33 verschieden lange auf Temperaturen von 45° bis 80° C. erwärmt wurde, 45° und 50° C. erhöhten bei kurzer Einwirkung zunächst die hemmende Wirkung des Extraktes, dann aber trat nach 1 Stunde wieder ein Rückgang ein. Bei Erwärmen auf 55°, 65° und 70° C. waren die Extrakte nach 30 minuten unwirksam geworden, während Erwärmen auf 80° C. schon in 10 Minuten inaktivierte.

Während in diesem Versuch der Extrakt bereits nach 30 Minuten bei 55° C. inaktiv wurde, zeigte sich bei anderen Extrakten von Rinderlunge eine Inaktivierung erst bei 63° C. Dies war aber die Temperatur, bei der Rinderlungenextrakte nach 30 Minuten stets wirkungslos wurden.

Analog reagierten die Extrakte aus den Lungen von weissen Mäusen, von schwarz-weissen Ratten von Hund und Affe: Bei 60—63° C. wurde jegliche Inhibinwirkung nach 30 Minuten zerstört.

Auch die Inhibine in Kaninchenlungenextrakt wurden bei 60—63° C. zerstört, doch zeigte sich einmal, dass der Lungenextrakt eines Tiere bei 60° C. noch nicht völlig zerstört war.

Lungenextrakte von Meerschweinchen schienen noch empfindlicher zu sein, denn in 7 Versuchen wurde die Inhibinwirkung schon nach 30 Minuten Erwärmen auf 56° C. aufgehoben und in 12 Versuchen nach 15 Minuten Erwärmen auf 63° C. Auch die Wirkung von Reisvogel-Lunge wurde nach 30 Minuten Erwärmen auf 56° C. aufgehoben.

Ganz anders verhielten sich in jedem Fall Extrakte aus frischen Menschenlungen, gleichgültig ob sie von ebengeborenen Kindern oder von Erwachsenen stammten. In der Tabelle 5 ist ein Versuch mit dem Extrakt von Kinderlunge C gegen die einfache, doppelte und vierfache agglutinierende Virusdosis nach Erwärmen auf 56°, 63° und 100° C. zusammengestellt. Erwärmen auf 56° C. steigerte die Wirkung, 63° und ebenso 80° C. (in der Tabelle nicht aufgenommen) war ohne jeden Einfluss, und erst 30 Minuten Erwärmen auf 100° C. schwächte etwas ab. Doch war hier noch deutliche Inhibinwirkung zu erkennen.

Während also alle anderen untersuchten Inhibine deutlich thermolabil waren, zeigte sich bei Menschenlunge eine ausgesprochene Thermostabilität. Es wurde bald deutlich, dass diese Thermostabilität nur bei frischen Menschenlungen nachweisbar war. Je

Tabelle 5.

Thermostabilität des Extraktes aus Kinderlunge C.

Erwärmung des Extraktes	Multiplum der agglutinierenden Dosis	Verdünnung des Lungenextraktes						
		1/25	1/50	1/100	1/200	1/400	1/800	1/1600
nativ	1×	0	0	0	+	+	+	+
	2×	0	0	0	+	+	+	+
	4×	0	+	+	+	+	+	+
56° C. 30′	1×	0	0	0	0	0	0	0
	2×	0	0	0	0	0	+	+
	4×	0	0	0	+	+	+	+
63° C. 30′	1×	0	0	0	0	+	+	+
	2×	0	0	+	+	+	+	+
	4×	0	+	+	+	+	+	+
100° C. 30′	1×	0	0	0	0	+	+	+
	2×	0	0	+	+	+	+	+
	4×	+	+	+	+	+	+	+

Tabelle 6.

Einfluss des Alterns der Lunge im Eisschrank auf die Thermostabilität des Lungenextraktes.

Aufenthalt der Lunge bei 4° C.	Hemmung der Haemagglutination durch das Multiplum der agglutinierenden Dosis	Lungenextrakt erwärmt auf			
		nativ	63° C. 30′	80° C. 30′	100° C. 30′
4 Tage	1×	1/400	1/400	1/100	100
	2×	1/400	1/100	1/25 nicht	1/25 nicht
	4×	1/200	1/50	1/25 nicht	1/25 nicht
5 Tage	1×	1/400	1/400	1/25 nicht	1/25 nicht
	2×	1/400	1/200	1/25 nicht	1/25 nicht
	4×	1/200	1/25	1/25 nicht	1/25 nicht
27 Tage	1×	1/200	1/200	1/25 nicht	
	2×	1/100	1/25 nicht	1/25 nicht	
	4×	1/50	1/25 nicht	1/25 nicht	

länger die Lunge im Eisschrank bei 4° C. oder im Frigolo bei —15° C. bewahrt wurde, je mehr lief die Thermostabilität zurück, um schliesslich vollkommen zu verschwinden. Ein Beispiel hierfür ist in Tabelle 6 gegeben. Nach einer Aufbewahrungszeit von 27 Tagen bei —15° C. waren die Inhibine in der Menschenlunge genau so thermolabil, wie die untersuchten Inhibine aus den verschiedenen Tierlungen. Da kaum vorzustellen ist, dass ein und dieselbe Substanz von Thermostabilität nach Thermolabilität umschlagen sollte, geht man wohl nicht fehl, wenn mann in der Menschenlunge zwei verschiedene Inhibine annimt: Ein thermostabiles, aber nur kurz haltbares, und ein thermolabiles, aber lange haltbares Inhibin.

In verschiedenen Fällen konnte man die Beobachtung machen, dass die in toto bei 4° C. aufbewahrte Lunge nach einigen Tagen in den daraus bereiteten Extrakten stärkere Inhibinwirkung zeigte als im frischen Zustand. Ein Beispiel hierfür findet sich in Tabelle 7. Später nahm die Wirkung wieder etwas ab.

Tabelle 7.

Einfluss des Alterns auf die hemmende Wirksamkeit von Lungenextrakt.

Cavialunge	Multiplum der agglutinierenden Dosis	Agglutination bei Verdünnung des Lungenextraktes				
		1/30	1/60	1/120	1/240	1/480
frisch	1×	0	0	0	0	0
	2×	0	0	0	0	0
	4×	+	+	+	+	+
	8×	+	+	+	+	+
48h 4° C.	1×	0	0	0	0	0
	2×	0	0	0	0	0
	4×	0	0	0	0	0
	8×	0	0	0	+	+

Haltbarkeit der Lungenextrakte.

Die Haltbarkeit der Lungenextrakte war nicht besonders gut. Aus diesem Grunde wurden sie stets frisch hergestellt. Bei 4° C. war Rinderlungenextrakt nach 3 Tagen zwar manchmal noch gut wirksam, nach 8 Tagen aber schon sehr deutlich schwächer und nach 20 Tagen vollkommen wirkungslos. Bei —15° C. war der

gleiche Extrakt bis zu 8 Tagen unverändert, nach 20 Tagen aber erwies auch er sich als fast unwirksam.

Bindungsfähigkeit des Lungenextraktes.

Wird stärker Lungenextrakt mit Vaccinevirus gemengt, so wird das gebundene Virus für Haemagglutination ungeeignet, nicht verbrauchtes Inhibin aber bleibt in seiner Wirkungsfähigkeit erhalten. Als Beispiel sei folgender Versuch wiedergegeben: Fallende Mengen von Virus wurden mit der konstanten Menge Kaninchenlungenextrakt 1/25 gemengt und 2 Stunden lang stehen gelassen. Wie aus Tabelle 8 hervorgeht, war die hohe Virusdosis von 1/50 im ersten Röhrchen noch imstande zu agglutinieren, während die Virusdosen 1/100 bis 1/800 (Grenzdosis) nach 2 Stunden infolge Inhibinwirkung keine Agglutination zeigten. Von den 3 Kontrollröhrchen agglutinierte die Kaninchenlunge allein nicht, wohl aber die beiden Viruskontrollen ohne Lungenextrakt. Die überstehende Flüssigkeit wurde nunmehr abgegossen und auf noch vorhandene hemmende Wirkung in fallenden Dosen gegen eine Virusverdünnung von 1/200 untersucht. Die Flüssigkeit des ersten Röhrchens hemmte nicht, da alles Inhibin durch die hohe Virusdosis gebunden war. Die Flüssigkeit des 2. Röhrchen hemmte bis zur Verdünnung 1/2, aber die Flüssigkeiten aus dem 3.-5.Röhrchen hemmten die Haemagglutination genau so stark, wie die reine Lungenextraktkontrolle. Die überstehende Flüssigkeit aus dem Viruskontroll-Röhrchen hemmte nicht. Dass es sich in der Tat um die gewöhnliche Inhibinwirkung handelte, wurde durch die vollkommen fehlende Hemmung der auf 60° C. erhitzten überstehenden Flüssigkeiten bewiesen.

Inhibinwirkung von Extrakten nicht normaler Lungen.

Weiterhin wurde untersucht, wie sich die Inhibinwirkung verhielt, wenn die Extrakte nicht aus normalen, sondern aus Lungen stammten, die mit Keuchhustenbacillen, Pestbacillen oder Vaccinevirus infiziert waren.

14 Meerschweinchen wurden mit 10 Milliarden Pestbacillen (apathogener Stamm Tjiwidej) in 0,5 ccm phys. Kochsalzlösung in leichter Aethernarkose pulmonal infiziert. 4 Tiere wurden nach 24 Stunden, 3 nach 48 Stunden, 3 nach 72 Stunden und 3 nach 120

Tabelle 8.

Einfluss von Vorbehandlung mit Vaccinevirus auf die hemmende Wirkung von Kaninchenlungenextrakt.

No.	Virus	Extrakt aus Kaninchenlunge	Agglutination nach 2^h	Haemagglutination durch Vaccinevirus 1/200 bei Zusatz der überstehenden Flussigkeit in der Verdünning von					Ebenso nach Erwärmen der überstehenden Flüssigkeit 30′ auf 60° C.	
				1/1	1/2	1/4	1/8	1/16	1/1	
1	1/50	1/25	+	+	+	+	+	+	+	+
2	1/100	1/25	0	0	0	+	+	+	+	+
3	1/200	1/25	0	0	0	0	+	+	+	+
4	1/400	1/25	0	0	0	0	+	+	+	+
5	1/800	1/25	0	0	0	0	+	+	+	+
6	—	1/25	0	0	0	0	+	+	+	+
7	1/50	—	+	+	+	+	+	+	+	+
8	1/800	—	+	+	+	+	+	+	+	+

Stunden untersucht. Alle wiesen deutlich Inhibinwirkung auf, analog entsprechenden Kontroll-Meerschweinchen.

Ebensowenig Einfluss hatte auch die pulmonale Infektion von Meerschweinchen mit je 10 Milliarden Keuchhustenbacillen.

Interessant sind auch die Ergebnisse nach Infektion mit Vaccinevirus, wobei 0,5 ccm einer Virussuspension von 1/10 unter Aethernarkose pulmonal instilliert wurde. Die Ergebnisse sind in Tabelle 9 zusammengestellt. In diesem Versuch wurde vorher untersucht, ob mittels der Haemagglutinationsmethode in den Lungenextrakten Vaccinevirus nachweisbar war. Dies war bei einem Tier der Fall, das nach 2 Tagen, und ausserdem bei 2 Tieren, die beide am 5. Tage getötet wurden. Alle anderen Lungenextrakte waren nicht imstande Hühnerblut zu agglutinieren. Dagegen wiesen alle diese Lungen eindeutig hemmende Stoffe auf und zwar die am 1. und 2. Tage untersuchten etwas weniger stark als die später untersuchten.

Tabelle 9.

Hemmung der Haemagglutination durch Lungenextrakt und Virusnachweis in der Lunge mittels Haemagglutination bei pulmonal mit Vaccinevirus infizierten Meerschweinchen (0,5 ccm 1/10).

Tier No.	Untersucht nach Tagen	Hemmung der		Haemagglutination durch Lungenextrakt
		doppelten Virusdosis	vierfachen Virusdosis	
1	1	1/100	1/25	neg.
2	1	1/50	1/25	neg.
3	1	1/50	1/25	neg.
4	2	1/100	1/25	neg.
5	2	1/100	1/25	neg.
6	2	—	—	1/50
7	3	1/200	1/100	neg.
8	3	1/200	1/200	neg.
9	5	—	—	1/400
10	5	—	—	1/400
11	9	1/400	1/200	neg.
12	10	1/200	1/100	neg.
13	10	1/800	1/25	neg.
14	10	1/200	1/50	neg.

Aus den hier mitgeteilten Versuchen geht hervor dass die Inhibinwirkung auch vorhanden ist, wenn die Lungen durch nicht

tödliche Infektion mit Bakterien oder durch Vaccinevirus selbst beeinflusst sind.

Einfluss der Inhibine auf die Infektiosität von Vaccinevirus.

Eine Suspension von Vaccinevirus 1/10 wurde 10 Minuten lang bei 2000 Umdrehungen zentrifugiert und die überstehende Flüssigkeit wurde beginnend mit 1/500 in fallenden Verdünnungen mit der konstanten Menge 1/25 einer Suspension aus Rinderlunge verdünnt. Eine gleiche Virus-Verdünnungsreihe wurde mit physiologischer Kochsalzlösung als Kontrolle verdünnt. Beide Versuchsreihen wurden 2 Stunden lang bei 37° C. bewahrt und hierauf nach der Ginsschen Methodik am skarificierten Meerschweinchenauge geprüft. Am gleichen Meerschweinchen wurde rechts Virus + Inhibin und links die entsprechende Kontrolle untersucht, ausserdem wurden für jede Verdünnung je 2 Meerschweinchen gebraucht. Der gleiche Versuch wurde wiederholt, wobei das Gemisch Virus + Lungenextrakt und die Kontrollreihe erst 2 Stunden bei 37° C., dann 18 Stunden bei 4° C. und noch einmal 1 Stunde bei 37° C. bewahrt wurden. In beiden Versuchserien ergab die Virusverdünnung 1/100.000 mit und ohne Lungenextrakt ein positives Impfergebnis. Bei einem Tier war ausserdem die Kontrollimpfung mit 1/200.000 schwach positiv.

In genau der gleichen Weise verliefen Versuche, wobei Kaninchen mit 0,1 ccm Virus + 1/5 Lungenextrakt intracutan injiziert wurden. Virus + Lungenextrakt und Virus allein ergaben genau die gleichen Werte: Die Konzentrationen von 1/1.000.000 und 1/500.000 waren in jedem Falle gleichmässig positiv.

Aus diesen Versuchen geht eindeutig hervor, dass die Inhibine keinerlei Einfluss auf die Infektiosität des Vaccinevirus auszuüben imstande sind. Diese Schlussfolgerung ist zweifellos richtig, wenn uns auch im Verlauf von anderen Versuchen deutlich geworden ist, dass Bindungsversuche zwischen Vaccinevirus und Antistoffen sowohl im Meerschweinchenversuch (Skarifikation) als auch im Kaninchenversuch (i. cut.) nur mit der allergrössten Vorsicht zu bewerten sind. Im Meerschweinchenversuch kann die Streuung sehr gross sein, und man hat mit Minimalwerten zu tun. Dies ist zwar bei der Kontrolle von Vaccine keine Hinderniss, wohl aber bei Neutralisierungsversuchen. Dagegen sind die kleinen und schwachen javanischen Kaninchen infolge unverhältnismässig

grosser Unterschiede in ihrer Reaktionsfähigkeit völlig unbrauchbar.

Aufhebung der Inhibinwirkung durch Lungenantiseren.

Es wurden verschiedene Antiseren gegen Lungextrakt vom Kaninchen, Maus und Ratte untersucht. Da in der Regel zwecks Vermeidung von Tierverlusten und unspezifischer Faktoren nur an 6 aufeinander folgenden Tagen immunisiert wurde, erwiesen sich nicht alle Seren wirkungsvoll.

Tabelle 10.

Aufhebung der Inhibinwirkung verschiedener Lungenextrakte durch Anti-Mäuselungen-Serum IV*a*.

Lungenextrakt von	Anti-Mäuselungen-Serum	Multiplum der agglutinierenden Dosis	Lungenextrakt-Endkoncentration					
			1/80	1/160	1/320	1/640	1/1280	NaCl
Kaninchen	1/20	4×	+	+	+	+	+	+
		2×	0	0	0	+	+	+
	—	4×	0	0	+	+	+	+
		2×	0	0	+	+	+	+
Meerschweinchen (von 3 Tieren)	1/20	4×	+	+	+	+	+	+
		2×	+	+	+	+	+	+
	—	4×	0	0	+	+	+	+
		2×	0	0	+	+	+	+
Ratte (von 3 Tieren)	1/20	4×	+	+	+	+	+	+
		2×	+	+	+	+	+	+
	—	4×	0	0	0	0	0	+
		2×	0	0	0	0	0	+

In Tabelle 10 ist der Einfluss eines Anti-Mäuse-Lungen-Serums auf die hemmende Wirkung von Extrakten aus Lunge von Kaninchen, Meerschweinchen und Ratte angegeben. Die Endverdünnung des inaktivierten Antiserums betrug 1/20. Das Antiserum 1/5 0,2 ccm wurde mit der gleichen Menge fallender Verdünningen von Lungenextrakt gemengt und 30 Minuten lang bei 37° C. gebunden.

Eine Verdünnungsreihe von Lungenextrakt mit phys. Kochsalzlösung diente als Kontrolle. Hierzu wurden je 0,2 ccm Vaccinevirus gefügt und nach 30 Minuten bei 22° C. je 0,2 ccm 2 % Hühnerblutsuspension. Aus den Kochsalzkontrollen ist zu ersehen, dass dieses Antiserum keine eigene Hemmung auf die Virus-Haemagglutination ausübte. Dagegen wurde die Hemmungswirkung des Kaninchenlungenextraktes teilweise und die der Lungenextrakte von Meerschweinchen und Ratte vollkommen aufgehoben. Da dieses Anti-Mäuse-Lungen-Serum auch gegen die Inhibine aus den Lungen anderer Tierarten wirksam war, handelt es sich bei dessen neutralisierender Wirkung sicher nicht um species-specifische Antistoffe, vielmehr um echte Antistoffe gegen Inhibine. Diese scheinen demnach bei den verschiedenen Tierspecies wenn auch nicht identisch, so doch mindestens miteinander verwandt zu sein.

Tabelle 11.

Aufhebung der Inhibinwirkung von Kaninchenlunge durch absorbiertes Anti-Mäuse-Lungen-Serum IV.

Adsorption des Antiserums mit	Multiplum der agglutinierenden Dosis	Kaninchenlungenextrakt		
		1/20	1/40	1/80
—	4×	+	+	+
	2×	+	+	+
Kaninchenlunge	4×	0	0	0
	2×	0	0	0
Kaninchenleber	4×	+	+	+
	2×	+	+	+
Mäuseleber	4×	+	+	+
	2×	+	+	+
Hühnerblut	4×	+	+	+
	2×	0	0	0
Menschenblut	4×	+	+	+
	2×	+	+	+
Kein Antiserum	4×	0	0	+
	2×	0	0	+

Da manche Antiseren von den verschiedenen Tierarten spontane Agglutinine gegen Hühnerblut enthalten, die durch Adsorption an Hühnerblut und oftmals auch an Hammelblut entfernt werden können, wurden die gewonnenen Antiseren meistens erst nach erfolgter Adsorption untersucht. Daneben wurden auch Adsorptionen an andere Organsuspensionen untersucht, wovon folgendes Beispiel in Tabelle 11 gegeben sei.

Inaktiviertes Anti-Mäuse-Lungen-Serum IV wurde in der Verdünnung von 1/5 mit je 1 g Kaninchenlunge, Kaninchenleber, Mäuseleber oder je 1 ccm gewaschenen conc. Hühnenblutkörperchen oder Menschenblutkörperchen gemengt, 1 Stunde bei 37° C. gehalten und danach abzentrifugiert. Je 0,25 ccm wurde mit 0,25 ccm fallender Konzentrationen von Kaninchenlungenextrakt gemengt

Tabelle 12.

Aufhebung der Inhibinwirkung von Rinderlunge durch verschiedene Konzentrationen absorbierten Anti-Mäuse-Lungen-Serums 119.

Antiserum absorbiert mit	Endverdünnung des Antiserums	Multiplum der agglutinierenden Dosis	Rinderlungenextrakt-Endverdünnung					
			1/25	1/50	1/100	1/200	1/400	NaCl
Hühnerblut	1/20	4×	+	+	+	+	+	+
		2×	0	+	+	+	+	+
	1/40	4×	0	+	+	+	+	+
		2×	0	0	+	+	+	+
	1/80	4×	0	0	0	+	+	
		2×	0	0	0	0	+	
Hammelblut	1/20	4×	+	+	+	+	+	+
		2×	+	+	+	+	+	+
	1/40	4×	0	0	+	+	+	+
		2×	0	0	0	+	+	+
	1/80	4×	0	0	0	+	+	
		2×	0	0	0	+	+	
Kein Antiserum		4×	0	0	0	+	+	+
		2×	0	0	0	0	+	+

und 30 Minuten lang bei 37° C. gehalten. Hierauf wurden 0,25 ccm der zweifachen und vierfachen wirksamen Virusverdünnungen zugefügt und das Ganze wieder 30 Minuten lang bei 22° C. gehalten. Zum Schluss wurde 0,25 2%ige Hühnerblutsuspension beigegeben. Das nicht adsorbierte Antiserum hob die Inhibinwirkung auf. Unverändert war das Serum auch durch Vorbehandlung mit Kaninchenleber, Mäuseleber und Menschenblut. Hühnerblut hob die neutralisierende Wirkung teilweise auf und Kaninchenlunge vollkommen. In beiden Fällen handelt es sich nicht um eine wirkliche Aufhebung der Antiserumwirkung. Hühnerblut enthält vielfach ein schwach wirkendes Inhibin, und in der Kaninchenlunge war sehr viel Inhibin enthalten, sodass die Antiserumwirkung durch die bei der Vorbehandlung dazugekommene Inhibinmenge abgeschwächt bzw. gänzlich aufgehoben wurde.

Schliesslich ist in Tabelle 12 noch ein Beispiel gegeben, wie ein Anti-Mäuse-Lungen-Serum nach Adsorption an Hühner- und Hammelblut austitriert wurde. In den Verdünnungen von 1/20 war der Serumeinfluss sehr deutlich zu sehen, auch wirkte das Hühnerblut etwas abschwächend, im Vergleich mit dem Hammelblut. In beiden Fällen war die Verdünnung von 1/40 schon viel schwächer, und die Verdünnung von 1/80 wirkte ungefähr wie die nicht Serum enthaltenden Kontrollen des Lungenextraktes. Die Antiserumwirkung ist also zwar deutlich feststellbar, die benutzten Seren waren aber doch relativ schwäch.

Besprechung der Ergebnisse.

Bei der Untersuchung der Inhibinwirkung auf die Haemagglutination durch verschiedene Virusarten sind durch Beveridge und Lind (1), Bovarnick und de Burgh (2), de Burgh, Yu, Howe und Bovarnick (3), Burnet und Beveridge (4), Burnet, McCrea und Anderson (5), Friedewald, Miller und Whatley (8), Green and Wooley (10), Henle, Henle und Harris (12), Stone und Burnet (15), Svedmyr (16) u.a. die verschiedensten Extrakte und Stoffe als wirksam gefunden. Wichtig sind in diesem Zusammenhang vor allem die Hemmungen der Influenza- und Mumps-Haemagglutination durch Extrakte aus menschlicher Lunge, Leber, Niere und Milz und durch Organextrakte von normalen Kaninchen und Meerschweinchen. Die Wirksamkeit derartiger Organextrakte wurde durch Erwärmen auf 65° C. reduziert.

Inhibinwirkung gegen Vaccinevirus wurde bisher nur in roher

Kälberlymphe durch Stone und Burnet (15) nachgewiesen.

Wenn bisher auch noch nicht viel über die Natür der Inhibine bekannt ist, so bekommt man doch den Eindruck, dass hier zwei verschiedenen Typen vorliegen. Die Inhibine der einen Gruppe, die möglicherweise für den Infektionsmechanismus wichtig sind und als Virus-Acceptoren angesehen werden können, stellen anscheinend einen normalen Bestandteil mit bestimmten Funktionen im Lebensablauf bestimmter Zellen dar. In dieser Eigenschaft muss ein solcher Acceptor mindestens zum grossen Teil aus Proteinsubstanzen bestehen und ist jedenfalls wegen dieser seiner Eiweissnatur auch mehr oder weniger ausgesprochen thermolabil. Eine zweite Gruppe von Inhibinen hat jedenfalls einen anderen Aufbau, vorwiegend von Nicht-Eiweisstruktur, wodurch seine Thermostabilität zu erklären wäre. Hierher würde beispielsweise der von Svedmyr (16) in normaler Allantoisflüssigkeit nachgewiesene Inhibitor gegen Grippevirus gehören, der erst nach 1 Stunde Kochen eine gewisse Abschwächung erfährt. Auch der in den vorliegenden Versuchen in Menschenlunge gefundene thermostabile Inhibinfaktor darf hierher gerechnet werden, wie auch die verschiedenen Inhibitoren der Influenze-Haemagglutination (Flachssaat-Gummi, Pectine, Akazien-Gummi usw.) von Green und Wooley (10) und die ebenso wirkende Blutgruppen A- und O-Substanz nach Burnet, McCrea und Anderson (5).

Man darf annehmen, dass ein in Organextrakten nachweisbarer Inhibitor, der den Viruspartikelchen die Bindung an empfängliche Erythrozyten möglich macht, auch im lebenden Organismus vorhanden ist und *in vivo* besondere virusaffine Eigenschaften besitzt. In Wirklichkeit liegt also ein Virus-Acceptor vor, der das in den Körper eingedrungene Virus bindet.

Diese Verankerung an die Acceptoren der Lunge kann keineswegs den Zweck haben, das Virus unschädlich zu machen. Die Acceptoren haben nichts zu tun mit Serum-Antistoffen, sondern wirken vollkommen unabhängig von diesen. In vaccineimmunisierten Tieren, die einen mässig hohen Serumtiter aufweisen, lässt sich die hemmende Wirkung des Lungenextraktes durch Erhitzen auf 56° und 63° C. zwar verringern, aber nicht völlig zum Verschwinden bringen. Es bleiben nach Inaktivierung der Acceptoren immer noch die spezifischen Antistoffe übrig, die ebenfalls die Haemagglutination hemmen. Schliesslich wurde auch in den Virus-Neutralisierungsversuchen gezeigt, dass die Acceptoren nicht imstande sind

die Infektiosität von Vaccinevirus nur im geringsten Masse zu beeinflussen.

Die Bedeutung der Acceptoren ist jedenfalls in etwas anderem zu suchen: Das Angehen der Infektion wird gewährleistet. Bei der Variola-infektion sind die Atemwege die wichtigste porte d'entrée. Die Acceptoren für das eingedrungene Virus finden sich in den Epithelien der Atemwege. Wenn die hier geschilderten Versuchen vorwiegend mit Lungenextrakten durchgeführt sind, so liegt das daran, dass mit diesen besonders gut quantitativ zu arbeiten ist. Abgeschabtes Trachealepithel wirkte gleichfalls agglutinationshemmend. Für eine besondere Affinität des Epithels der Atemorgane spricht auch der durch Gins, Hackenthal und Kamentzewa (9) geführte Nachweis von Virus auf den Schleimhaut der oberen Luftwege von Kindern 3—5 Tage nach der Erstvaccination und ebenso bei cutan, intracutan und intravenös infizierten Kaninchen und Meerschweinchen.

Gegenüber der Bedeutung der Atmungsorgane für das Zustandekommen der Infektion tritt die Haut an Wichtigkeit zurück. Es wäre sonst in früheren Zeiten infolge der damals geübten Variolation viel häufiger zu echten Variolaerkrankungen gekommen. Es ist daran zu denken, dass in der Haut neben Acceptoren noch andere die Virushaemagglutination hemmende Stoffe vorhanden sind, denn Stone und Burnet (15) fanden Anweisungen dafür, dass in Kalbslymphe eine Art Antistoff anwesend war, der den Serum-Antistoffen ähnelte.

Wenn auch in den Atmungsorganen zweifellos besonders viele Acceptoren für Pockenvirus vorhanden sind, so finden sich solche ebenfalls in anderen Organen, allerdings nicht so regelmässig und nicht in gleicher Menge. Dies stimmt überein mit den früheren Befunden über den Nachweis von Vaccinevirus in den inneren Organen. Unabhängig von der Infektionsweise wiesen Haagen und Kodama (11) bei Kaninchen und Mäusen in fast allen Organen Elementarkörperchen nach, nachdem schon vorher Bijl und Frenkel (6), Paschen (14) und Douglas und Price (7) Metastasen in fast allen Organen gefunden hatten. Schwieriger war der Nachweis in den Organen geimpfter Rinder, worauf Zeller, Gildemeister und Hilgers (17) und Lehmann (13) hinwiesen.

Antisera gegen Lungenextrakt vermögen die Inhibinwirkung aufzuheben. Diese Wirkung erstreckt sich nicht allein auf den Lungenextrakt, mit dem die Antiseren gewonnen sind, sondern auch auf

Lungenextrakte von ganz anderen Tierarten. Daher ist bei diesem Neutralisierungseffekt die species-specifische Komponente auszuschliessen. Auch Adsorption an anderes Gewebe, beispielsweise Lebergewebe, von der gleichen Tierart hebt die Wirkung nicht auf. Es ist auf eine gewisse Verwandtschaft der verschiedenen Vaccinevirus-Acceptoren in den Lungen der verschiedenen Tierspecies zu schliessen.

Wenn man sich auch besonders auf dem Gebiete der Virusforschung vor Verallgemeinerungen hüten muss, so liegt doch der Gedanke nahe, dass die Empfänglichkeit verschiedener Tierspecies für eine natürliche Infektion mit einem bestimmten Erreger abhängig sein dürfte von gleichen oder ähnlichen empfänglichen Acceptoren an den Zellen jener Organsysteme, die für diesen Erreger die porte d'entrée darstellen.

Zusammenfassung.

In dem Lungenextrakt verschiedener Tierspecies findet sich ein Stoff, der die Vaccine-Haemagglutination hemmt. Dieses Inhibin ist thermolabil und wird bei 56° oder 63° C. nach 30 Minuten zerstört. In der Lunge des Menschen findet sich daneben noch ein thermostabiler Stoff, der im Eisschrank schnell verschwindet.

Durch Immunisierung mit Lungenextrakt lässt sich vom Kaninchen Antiserum gegen die Inhibine nicht nur aus Mäuselunge, sondern auch gegen solche aus Lungen anderer Tierarten gewinnen.

Es wird die Bedeutung dieser die Haemagglutination hemmenden Stoffe als „Virus-Acceptoren" besprochen, die jedenfalls für das Zustandekommen der Infektion von Bedeutung sind.

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NEWER DIAGNOSTIC PROCEDURES IN TOXOPLASMOSIS

by

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In toxoplasmosis neutralizing antibodies could be demonstrated with SABIN and OLITSKY's „rabbit skin test". By means of a slightly modified test, we (8) examined in this way sera of 88 human beings, divided into 4 groups:

a. proven cases of toxoplasmosis (9);

b. 34 selected cases described by BINKHORST (1, 8) on account of eye-disturbances, and their mothers;

c. some cases in which the clinical symptoms furnished no sure proof of toxoplasma infection;

d. some persons, taken at random.

Our rabbit skin tests were evaluated by a statistical method which produced unequivocal results. We were aware of the fact that in this way some slightly positive sera have been considered as negative. Further objections were the loss of a rabbit in almost every experiment, and the long interval before the time of reading (one week following the intradermal injections).

Until the description of a new dye test in the diagnosis of toxoplasmosis by SABIN and FELDMAN (5), the neutralizing antibodies were considered to be heat labile and present in the serum in a very small quantity. Thus it was necessary to examine a serum sample as soon as it was prepared; otherwise it had to be mailed in a vacuum-flask with dry ice and stored in a dry ice box until examination. As a serum dilution as low as 1 : 10 already led to a negative result, the undiluted serum had to be used mixed with various dilutions of toxoplasma infected mouse brain suspension before the intradermal injections into the rabbit skin.

A completely new principle in demonstrating neutralizing antibodies has been put forward by SABIN and FELDMAN (5). The peritoneal exudate of mice, 3—4 days previously infected intraabdominally with a large dose of toxoplasma, is mixed with a fresh human serum and incubated in a waterbath of 37° C. for one hour. Then, a loopful of fluid is placed on a slide, mixed with an alkaline methylene blue solution [1]) and the toxoplasma are microscopized under a coverglass.

In the case of a serum, containing toxoplasma antibodies, the cytoplasma of the protozoa remains unstained (the nuclear substance may be stained) whereas, when a completely negative serum had been used, the cytoplasma of the toxoplasma should stain a deep purple blue.

The second important discovery of SABIN and FELDMAN (5) was the fact that the specific toxoplasma antibody is heat-stable and may be present in a high titer in human sera. For the completion of the immunity phenomenon occurring in the dye test an accessory factor, an activator-like substance not identical with complement, is necessary. This activator, present in almost every fresh human serum at a low titer — a serum dilution of $> 1 : 2$ is enough to inhibit its action — is heat-labile, and storing a serum at room temperature for a few hours is enough to inactivate the factor completely.

The specific antibody, according to SABIN and FELDMAN, can combine with the toxoplasma without any deleterious effect upon the parasites, the activating factor on the other hand is capable of combining with the parasites only in presence of the specific antibody. Only the combination of toxoplasma-specific antibody + activator will result in real neutralization or, to quote SABIN and FELDMAN, loss of the affinity of the cytoplasma for the dye is an index of death.

It is possible, with this new dye test, to estimate the 50 per cent endpoint titer of toxoplasma antibodies in a serum; for example, titers of 1 : 4096 were reached in experimentally infected monkeys, guinea pigs and rabbits.

Dr SABIN kindly mailed us the manuscript of his paper, some

[1]) 3 ml saturated alcoholic methylene blue solution + 10 ml of a buffer pH = 11, prepared in the following way: 9.73 ml 0.53 % Na_2CO_3 + 0.27 ml 1.91 % $Na_2B_4O_7 . 10 H_2O$. The mixture of dye and buffer can only be kept for a few days.

months before publication, thus abling us to study his new method at once. At first we had some difficulties in reading the test, so initially we combined some principles of the dye test with the rabbit skin test. Some examples are given here:

Experiment I.

Fresh serum of a young man with bilateral central chorio-retinitis; for 4 weeks the patient suffered from pneumonia and we wonder whether this lung-affection may have been of toxoplasmic origin.

	Mixture injected into the rabbit's skin after	
	½ h at room temperature	1 h in water bath of 37° C.
Control: toxoplasma suspension + Tyrode	14½	13½
Case: item + patient's serum	10	7½

This example shows a distinctly favourable influence of the higher temperature. The figures represent the sum of the values of 5 skin lesions of mixtures of the patient's serum with different dilutions of toxoplasma suspension (end dilutions 1 : 20—1 : 60 — 1 : 80 — 1 : 540 — 1 : 1620); each skin lesion is evaluated between 4—0 (maximum reaction with redness, swelling and central necrosis = 4 or ++++).

This experiment shows a difference of 4½ between the check and the patient's serum, when the mixture is kept at room temperature, and a difference of 6, when it is kept at 37° C. for one hour. The influence of the higher temperature has been checked in a number of tests, and its favourable effect was confirmed.

Experiment II.

Subsequently we examined a number of sera that presumably had been inactivated by the time factor.

Serum of an infant with hydrocephalia, progressive bilateral chorio-retinitis but without cerebral calcifications.

A. Without activator:

	½ h room temp.	1 hour water bath 37° C.
Control:	12	11
Patient's serum	11½	10

In this case the favourable influence of the higher temperature is not marked, possibly because the serum, considered as negative, did not contain a sufficient amount of activator substance.

B. With activator substance: fresh human serum that did not contain toxoplasma antibodies (when necessary stored in a dry ice box).

	½ h room temp.	1 hour water bath 37° C.
Control:	14	14
Patient's serum:	9½	8½

The serum was now considered as positive. The child died about 2 months later and we were informed by Prof. ULLRICH (Bonn), that microscopical examination of the lungs revealed many pseudocysts (preliminary communication).

In the meantime we continued our experiments with the dye test. We were advised by Prof. GARD (Stockholm) to mix the alkaline methylene blue with the mixture of the patient's serum, activator serum and toxoplasma suspension, as soon as the tubes were taken out of the water bath. Furthermore, we simplified the dye test in the following way, obtaining very satisfactory results.

Tube 1: 2 drops of patient's serum (undiluted, not-inactivated) + 4 drops of activator serum + 2 drops of toxoplasma suspension (by SABIN and FELDMAN's method we tried to obtain a sufficient amount of exudate on the third or fourth day following the intraperitoneal infection of the mice; the exudate was placed in a tube containing 1—2 drops of heparin solution, 1 : 100 and used within 1 hour).

Tube 2: 2 drops of patient's serum 1 : 5, further as in tube 1.

Tube 3: 2 drops of patient's serum 1 : 25, further as in tube 1.

Preparation of the patient's serum dilution: 2 drops of patient's serum + 8 drops of activator serum → dilution 1 : 5; one drop of dilution 1 : 5 + 4 drops of activator serum → dilution 1 : 25.

The end dilutions of the patient's serum, before placing them in the water bath, are: 1 : 4 — 1 : 20 — 1 : 100. As it is rather straining to microscopize in each serum dilution 50 toxoplasma, especially when most of them are unstained, we did not use higher serum dilutions.

Tube 4: 4 drops of activator serum + 4 drops of toxoplasma suspension.

Tube 5: 4 drops of Tyrode solution + 4 drops of toxoplasma suspension.

After the incubation in the water bath, we added to each tube 4 drops of alkaline methylene blue, mixed well and stored the tubes in the refrigerator until examination. As a rule we examined the toxoplasma immediately after the dye had been admixed, repeating the test the next day, after storage of the tubes in the refrigerator, and after shaking them thoroughly. The results of both counts were always approximately the same.

For all the tests mentioned in this paper we used the activator serum of one of ourselves, which was known to possess no toxoplasma antibodies.

We did not determine exactly the 50 per cent endpoint titer but regarded as positive a serum dilution giving about 50 per cent stained, respectively 50 per cent unstained toxoplasma. We did not ascertain the so called prozone effect (SABIN and FELDMAN observed this phenomenon in some strongly positive sera in which, up to a dilution 1 : 16, there was no inhibition of the cytoplasma staining).

We examined with the new dye test some sera which we had examined earlier in the rabbit skin test. The results are given in table I as positive or negative (8), but, owing to a slight modification the values of the modified test have not been evaluated statistically so far, so we had to record some sera as equivocal.

We regard the new dye test to be of the greatest value in the study of toxoplasmosis, especially as, in our mind, a strongly positive result ($> 1 : 100$) will confirm a clinical diagnosis; but, like the WASSERMAN test in syphilis, its value is limited and it may never be judged conclusive without clinical examination and laboratory records of the suspected person and, if possible, of his siblings. We do not feel, with the dye test, any need to perform FRENKEL's intradermal toxoplamin test, but nevertheless we prepared the reagent and a control reagent, according to his prescription (2) and examined some persons in this way as well as with the dye test. We expressed the skin reaction as $\pm$, + etc. up to + + + + but feel that this is not very exact (See Table II).

N°.	Serum	Rabbit skin test	st. = stained; unst. = unstained						Titer	Remarks
			Serum 1 : 4		Serum 1 : 20		Serum 1 : 100			
			st.	unst.	st.	unst.	st.	unst.		
1	Mother W.	pos.	8%	92%	12%	88%	22%	78%	> 1 : 100	—
2	Baby W.	pos.	25%	75%	30%	70%	34%	66%	> 1 : 100	Degenerative fundus lesions, O.D. diffuse retinitis, O.S. coloboma maculae.
3	Mother K.	—	62%	38%	90%	10%	100%	0%	0	—
4	Mr. K.	pos.	34%	66%	28%	72%	46%	54%	> 1 : 100	First slight suspicion of toxoplasmosis because of leucaemic blood picture with relative monocytosis afterwards diagnozed as sarcomatosis. Exitus, microscopical examination of tissue sections not yet performed. Intracerebral and intraperitoneal infection with a brain suspension, about 30 hours old, negative.
5	Mother Mr.	neg.	30%	70%	44%	56%	70%	30%	> 1 : 20	—
6	Baby Mr.	pos.	—	—	—	—	—	—	—	Hydrocephalia, fundus lesions, Exitus. Tissue sections especially of the lungs, yielded on microscopical examination a lot of toxoplasma pseudocysts.
7	Child Mst.	neg.	94%	6%	88%	12%	94%	6%	0	Neurological symptoms.
8	Marijke Fs.	—	80%	20%	88%	12%	88%	12%	0	Congenital blindness, fundus lesions.
9	Annie Cr.	—	24%	76%	26%	74%	30%	70%	> 1 : 100	O.S. retinitis during 1 year.
10	Mother A.	—	12%	88%	42%	58%	80%	20%	> 1 : 200	—
11	Baby A.	—	88%	12%	84%	16%	100%	0%		Fundus lesions, seizures, cong. vitium cordis.
12	Miss K.	neg.	44%	56%	60%	40%	86%	14%	> 1 : 4	Old chorio-retinitis centralis O.D.; debilitas.
13	Mrs. van S.-B.	pos.	28%	72%	41%	59%	60%	40%	> 1 : 20	O.S. focal lesion in fundo. See BINKHORST (1) case 13.
14	Baby Z.	—	48%	52%	85%	15%	90%	10%	1 : 4	Oligophrenia, chorea.
15	Mother H.	—	8%	92%	38%	62%	48%	52%	1 : 100	
16	Sister A. H.	—	46%	54%	60%	40%	88%	12%	1 : 4	Healthy.
17	Sister B. H.	—	32%	68%	32%	68%	48%	52%	1 : 100	
18	Son H.	pos.	24%	76%	46%	54%	84%	16%	1 : 20	O.O. old chorioiditis. A twinbrother, who was blind and paralyzed, died at the age of 8 years.
19	Mrs. Hs.	pos.	15%	85%	33%	67%	43%	57%	> 1 : 100	O.S. chorioiditis, O.D. papil edema. Slight atrophia cerebri, hypertension.
20	Mother Hn.	pos.	26%	74%	33%	67%	42%	58%	> 1 : 100	—
21	Baby Hn.	pos.	—	—	—	—	—	—	—	O.D. microphthalmia, coloboma N. optici; O.S. coloboma retinae. Ventricle system enlarged.
22	Mr. Br.	equiv.	24%	76%	56%	44%	80%	20%	> 1 : 4	Epilepsia. Calcium shadow in cerebro.
23	Mother Nr.	pos.	—	—	—	—	—	—	—	—
24	Baby Nr.	pos.	18%	82%	20%	80%	30%	70%	> 1 : 100	Bilateral microphthalmia, iritis, chorioretinitis. Calcium shadows in cerebro. Leftsided internal hydrocephalia. See BINKHORST (1) case 8.
25	Girl A. B.	—	96%	4%	96%	4%	96%	4%	0	Microcephalia. No calcium shadows in cerebro, no chorio-retinitis.
26	Mother K.	neg.	100%	0%	100%	0%	100%	0%	0	—
27	Boy K.	neg.	94%	6%	98%	2%	100%	0%	0	O.O. Chorio-retinitis scars. Slight atrophia papillae.
28	Mr. J.	neg.	40%	60%	66%	34%	88%	12%	1 : 4	One-sided oedema papillae, one sided Horner, pleocytosis of the lumbar fluid.
29	Mother v. d. L.	neg.	—	—	—	—	—	—	—	—
30	Baby v. d. L.	pos.	40%	60%	46%	54%	48%	52%	1 : 100	O.O. Large chorio-retinitis lesion. Calcium shadows in cerebro. See BINKHORST (1) case 4.
31	Mother van E.	pos.	40%	60%	40%	60%	34%	66%	> 1 : 100	
32	Father van E.	pos.	—	—	—	—	—	—	—	
33	Ada van E.	neg.	—	—	—	—	—	—	—	Healthy.
34	Jantje van E.	neg.	—	—	—	—	—	—	—	
35	Mary van E.	pos.	—	—	—	—	—	—	—	O.O. Large chorio-retinitis lesions. Neurological and mental disorders.
36	Mother D.	neg.	88%	12%	98%	2%	98%	2%	0	—
37	Child D.	neg.	—	—	—	—	—	—	—	Nervous disorders. No fundus lesions.
38	Mk. Br.	pos.	16%	84%	50%	50%	86%	14%	1 : 20	O.O. Chorio-retinitis centralis.
39	Mr. Ms.	neg.	34%	66%	50%	50%	94%	6%	1 : 20	O.S. Old and fresh chorio-retinitis lesions.
40	Mr. van C.	neg.	96%	4%	94%	6%	94%	6%	0	Reason of examination unknown to us.
41	Child M.	neg.	88%	12%	84%	16%	96%	4%	0	Luetic fundus lesions but WASSERMAN negative.
42	Mr. de Z.	equiv.	10%	90%	40%	60%	86%	14%	> 1 : 20	Reason for examination unknown to us.
43	Mother v.d. V.	pos.	18%	82%	20%	80%	78%	22%	> 1 : 20	—
44	Father v.d. V.	neg.	56%	44%	90%	10%	96%	4%	0	—
45 I	Child v. d. V.	pos.	20%	80%	32%	68%	86%	14%	> 1 : 20	Enucleatio bulbi because of suspicion of tumor in fundo;
45 II	item	—	84%	16%	88%	12%	98%	2%	0	on microscopical examination chorioiditis.
46	Mother R.	pos.	24%	76%	50%	50%	70%	30%	1 : 20	—
47	Child R.	equiv.	60%	40%	76%	24%	76%	24%	0	O.O. extensive chorio-retinitis. Hydrocephalia.
48	Mr. de V.	pos.	16%	84%	18%	82%	60%	40%	> 1 : 20	O.O. Chorio-retinitis centralis; 4 weeks previously the patient suffered from a pneumonia!
49	Mr. Br.	pos.	26%	74%	28%	72%	58%	42%	> 1 : 20	Eye disturbances.
50	M. Df.	neg.	80%	20%	86%	14%	90%	10%	0	Atypical pneumonia, trigeminus neuralgia.
51	Baby v. d. H.	neg.	64%	36%	96%	4%	100%	0%	0	Hydrocephalia.
52	Mr. Bs.	equiv.	20%	80%	76%	24%	94%	6%	1 : 4	O.O. retinitis centralis serosa.
53	Child v. d. H.	neg.	96%	4%	94%	6%	98%	2%	0	Reason for examination unknown.
54	Mother J.	—	96%	4%	96%	4%	98%	2%	0	—
55	Baby J.	—	98%	2%	98%	2%	100%	0%	0	Hydrocephalia. Several relations with big heads and debilitas.
56	Mother van O.	—	20%	80%	28%	72%	44%	56%	> 100	—
57	Baby van O.	—	84%	16%	90%	10%	92%	8%	0	Degenerative lesions in the retina.
58	Mother de R.	—	46%	54%	92%	8%	98%	2%	1 : 4	—
59	Baby de R.	—	68%	32%	86%	14%	98%	2%	0	Congenital microphthalmus and debilitas.
60	Mother B.	—	100%	0%	100%	0%	100%	0%	0	Healthy. In 6th month of pregnancy during one week
61	Father B.	—	34%	66%	46%	54%	86%	14%	1 : 20	headache and slight fever. At present these 3 persons are
62	Boy B.	—	92%	8%	98%	2%	96%	4%	0	quite healthy.
63	Baby B.	—	84%	16%	92%	8%	94%	6%	0	Congenital hydrocephalia, fundus lesions, no calcium shadows. Spinal fluid xanthochrome, with a high percentage of albumen.
64	Miss C. M.	—	30%	70%	48%	52%	86%	14%	1 : 20	Atrophic and pigmentary chorio-retinitis; first attack 4 years ago, second attack last year, the third attack at present. General state of patients health florid. She lives on a farm in N. Africa.
65	Boy v. d. W.	—	96%	4%	94%	6%	96%	4%	0	Hydrocephalia.
66 I	Miss B.	—	46%	54%	78%	22%	88%	12%	1 : 4	
66 II	item	—	48%	52%	36%	64%	84%	16%	> 1 : 20	Chorio-retinitis (macular lesions).
67	Baby de W.	—	50%	50%	80%	20%	90%	10%	1 : 4	Seizures.
68	Boy Th.	—	44%	56%	76%	24%	90%	10%	1 : 4	Hydrocephalia.

Remarks:

We performed the new dye test on 68 human sera; on 43 sera we performed the rabbit skin test and some time later we examined 37 of these sera with SABIN and FELDMAN's test: of 15 sera with a negative rabbit skin test, 11 showed also a negative dye test, 1 a titer of 1 : 4, 1 a titer of > 1 : 4, 1 a titer of 1 : 20 and only 1 a titer of > 1 : 20. According to SABIN and FELDMAN, a titer of 1 : 16 usually corresponds to a negative rabbit skin test. 5 sera with an equivocal result in the rabbit skin test showed titers of: 0 — 1 : 4 — > 1 : 4 — > 1 : 20 — > 1 : 20. From 17 sera with a positive rabbit skin test, 8 showed a titre of 1 : 100 or more, the other 9 a titer of 20 or < 20. We may therefore conclude that the new dye test is a more accurate, valuable diagnostic test in toxoplasmosis, cheaper and quicker to perform than the rabbit skin test, never giving equivocal results.

We examined 17 serum pairs of mother and child: 8 serum pairs of mother and infant showed the following combinations: > 100 + > 100 — < 20 + 0 — 0 + 0 — > 20 + > 20 — > 100 + 0 — 4 + 0 — 0 + 0 — 0 + 0 (father 1 : 20). Of 6 serum pairs of mother and infant the dye test has been performed only on one serum, no more of the corresponding serum being available; so the result of the rabbit skin test (r. s. t.) is given: > 20 + r. s. t. pos. — > 100 + r. s. t. neg. — r. s. t. pos. + > 100 — r. s. t. neg. + 100 — 100 + r. s. t. pos. — 0 + r. s. t. neg. Three serum pairs concerned mother and adult child, in which, of course, an acquired toxoplasmic infection is not disclosed: 0 + > 100 — 100 + 20 (the 2 sisters of the patient: 1 : 4 resp. 1 : 100) — 20 + 0.

In one case (no. 45) we observed the first time a positive rabbit skin test and, in the same serum, a titer of > 1 : 20 (no. 45 I). The serum of the mother (no. 43) showed a similar result. After about six months, fresh serum of the mother remained still > 1 : 20, but the fresh serum of the boy was now negative (no. 45 II). As it is assumed that antibodies to toxoplasma remain for years in the serum, we could not explain this drop.

TABLE II.

1. Miss F., healthy. Titer (dye test) before the intradermal test 1 : 4, after the injection 1 : 20. Toxoplasmin test +++, check 0. FRENKEL, not knowing of SABIN and FELDMAN's dye test, observed that human beings giving a negative rabbit skin test with their serum, may give a positive rabbit skin test when their serum is rechecked after the intradermal toxoplasmin injection. We observed here a rise in titer from 1 : 4 to 1 : 20.
2. Dr M., healthy. Serum before the toxoplasmin injection in the dye test as well as in the rabbit skin test negative. Reaction to toxoplasmin ±, in the control 0. Serum in the dye test, taken after the toxoplasmin injection, also negative. As toxoplasmin, like tuberculin, is not an antigen, it will not induce the formation of antibodies to toxoplasma, but is only capable of temporarily raising the level of the serum antibody content.
3. Mr de B. Dye test 0. FRENKEL's test 0. Dye test after intradermal reaction also 0.

   As in some cases, not all described here, the reaction to the toxoplasmin as well as to the control reagent was rather strong (allergy to mouse protein) we performed some intradermal tests with 3 dilutions of the toxoplasmin: 1 : 10, 1 : 100, 1 : 1000 and did the same, of course, with the control fluid:

4. Nos. 60—63 from table I. We give only our total impression of the reactions; the serum test following the intradermal test could not be performed as yet.
   - No. 60. Mother B. Titer 0. FRENKEL test with toxoplasmin 0, with control reagent 0.
   - No. 61. Father B. Titer 1 : 20. FRENKEL test with toxoplasmin +++, with control reagent ±.
   - No. 62. Boy B. Titer 0. FRENKEL test with toxoplasmin +±, with control reagent +±.
   - No. 63. Baby B. Titer 0. Here we injected, according to FRENKEL 0.1 ml of its own ventricle punctate intradermally, as in this body fluid slight traces of toxoplasma antigen may occur in cases of cerebral toxoplasmosis. The reaction was negative. We made the same observation in case No. 6 of table I.

Under circumstances in which toxoplasma are not available, FRENKEL's intradermal test may be of some value for the diagnosis of toxoplasmosis, but we prefer to perform SABIN and FELDMAN's new dye test.

Summary.

Practical experience with SABIN and FELDMAN's new dye test in toxoplasmosis is described. In 68 cases in which this test, somewhat modified and simplified, was performed, it has proved very

satisfactory. FRENKEL's intradermal toxoplasmin test, although very simple to perform, was less valuable.

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(From the Virus Laboratory of the University Medical Clinic, Leyden).

RAPID DIAGNOSIS OF THE SUB-GROUP OF INFLUENZA-A VIRUS STRAINS ISOLATED DURING THE EPIDEMIC OF 1949 IN THE NETHERLANDS

by

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(Received May 13, 1949).

By inactivating the non-specific inhibitor against influenza virus in ferret anti-sera, rapid diagnosis of the ,,sub-group'' of influenza-A virus strains can be arrived at, because the antibody pattern in the cross haemagglutination inhibition test appears very distinctly, also when using strains recently isolated in embryonated eggs. The sera are freed from non-specific inhibition by means of enzyme from *Vibrio cholerae* (MULDER and VAN DER VEEN (1)). We used the cholera strain Z4, kindly sent us by professor BURNET. Chicken cells and haemagglutination pattern-reading were used throughout all experiments.

Rapid diagnosis of the sub-group, using young egg-strains.

In our tests we employed the ferret sera: WS (1933 E), PR8 (1934 U.S.A.), A (1941 N) [2]), Barratt (1947 E) [3]) and Rhodes (1947 U.S.A.) [3]). These sera belong to strains which show a high titer with the homologous and the heterologous sera of the same sub-group.

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[1]) Sub-unit of the Influenza Research Team of the Institute of Preventive Medicine, Leyden. With the financial support of the Institute of Preventive Medicine, the Department of Medical Research Philips Roxane (Weesp), the State Department of Science, and the Jan Dekker Fund.

[2]) Isolated in the 1941-influenza epidemic in the Netherlands.

[3]) The first strains of this sub-group were found by ANDERSON and BURNET in Australia (1945 and 1946). In America this sub-group is provisionally labelled as A-prime.

(We do not possess any other representatives of the "sub-group" WS, but so far the strain would seem to represent a separate group).

A diagram of the possible immune patterns in cross-haemagglutination inhibition tests within one of the sub-groups of A-strains as obtained in our laboratory is as follows:

Diagram of antibody patterns within the same sub-group of influenza-A strains

Antiserum	Strain		
	P	Q	R
P	++++	+	++++
Q	+	+	++++
R	+	+	++++

+ = low titer; ++++ = high titer.

From this diagram it appears that R-strains are quite suitable to be used in cross tests. Among others they are represented by PR8 and A (1941 N) for pre-1947 strains (with the exception of WS) and by RHODES and BARRATT for 1947-strains. Q-strains are useless in one direction, but the homologous antiserum displays their serological pattern very well when R-strains are used. Nor are P-strains very suitable, as they are not so satisfactorily fixed by the heterologous antisera as by the homologous (VAN DER VEEN and MULDER (2)). If, therefore, one possesses only the young virus strain without its homologous antiserum, it is clear that difficulties may be encountered in the reading of the serological pattern, in case the young strain should give a low titer with antisera of the homogous and heterologous sub-groups.

From table 1 it follows that the serum titers against certain young egg-strains from the 1949 epidemic are generally low, but that the identification of the sub-group is possible, because the sera of the heterologous sub-groups do not show any titer against the strains. When working with the young egg-strains the agglutination patterns must be read immediately after settling, as they are sometimes rather unstable.

Rapid diagnosis of the sub-group, using ferret antisera of new strains.

Great certainty is obtained about the sub-group when the ferret

TABLE 1.

Haemagglutination-inhibition-tests with stock-sera against young egg-strains of influenza-A virus (1949 Nederland)

Pairs of antiserum (Ferret)	Strains					
	Hok (1949 N) AMN. 1[1])	Ende (1949 N) AMN. 1[1])	Sips (1949 N) AMN. 1[1])	Mark (1949 N) AMN. 2 ALL. 1[2])	Mas (1949 N) AMN. 2 ALL. 1[2])	Homologous strains[3])
WS (1933 E)	< 12/< 12	< 12/< 12	< 12/20	< 12/< 12	< 12/< 12	<12/ 8300
PR8 (1934 U.S.A.)	—	< 12/< 12	< 12/15	< 12/< 12	< 12/< 12	<12/13650
A (1941 N)	< 12/< 12	< 12/< 12	< 12/< 12	< 12/< 12	< 12/< 12	<12/ 8450
Barratt (1947 E)	—	< 12/640	< 12/340	< 12/340	< 12/340	<12/ 5490
Rhodes (1947 U.S.A.)	< 12/672	< 12/380	< 12/112	< 12/170	< 12/170	<12/ 8360

[1]) AMN. 1 = Amniotic fluid from first inoculated egg (titer at least 30).

[2]) AMN. 2 ALL. 1 = Allantoic fluid from first allantoic passage after two amniotic passages.

[3]) Mean of 3 experiments during the period of these investigations.

The titer of the sera is expressed as the reciprocal of the dilution which with 3-agglutination units of the virus gives a two-plus agglutination pattern (maximal agglutination pattern noted as four-plus). All sera freed from non-specific inhibitor.

antiserum of the new strains is used against the above-mentioned laboratory stock strains. The quickest results are obtained when one uses the ferret serum from an animal which has been inoculated with positive garglings, sputum, or autopsy material. A survey of such cases is given in table 2. In four cases out of six the diagnosis of the sub-group was possible eight days after the inoculation of the ferret. In all the six cases the serological pattern was clear after thirteen days, and in three out of them the titers of the sera were notably high.

All the A-strains (31) from the epidemic of the winter of 1949 in the Netherlands that were so far examined belonged to the same subgroup of influenza-A as the ,,1947''-strains. In final tests Lee-and Swine-is-antiserum were included.

Summary.

When ferret antisera are freed from non-specific inhibitor against influenza virus, rapid diagnosis of the sub-group of new strains can be arrived at by using young egg-strains, or by using

TABLE 2.

Haemagglutination-inhibition tests with sera of ferrets inoculated with garglings or trachea mucosa from patients with influenza.

The prae-infectious ferret-sera showed a titer of <12 against all the strains used.

Antisera (Ferret)	Strains						
	days	WS (1933 E)	PR8 (1934 U.S.A.)	A (1941 N)	Barratt (1947 E)	Rhodes (1947 U.S.A.)	Homologous strain
Hof (1949 N)	8	<12	<12	<12	84	47	373
(Garglings)	13	<12	14	86	2986	3548	2072
Burl (1949 N)	8	<12	<12	54	373	224	—
(Garglings)	13	<12	<12	48	5973	4778	—
Kraa (1949 N)	8	<12	<12	43	93	56	—
(Garglings)	13	<12	56	144	746	672	—
Oord (1949 N)	8	<12	<12	93	80	56	—
(Garglings)	13	<12	<12	126	320	298	—
Behr (1949 N)	8	<12	<12	<12	<12	<12	—
(Garglings)	13	<12	<12	14	576	672	—
Hee (1949 N)	9	<12	<12	<12	384	373	768
(Mucosa of Trachea)	13	<12	<12	<12	1536	2688	3072

The titer of the sera is expressed as the reciprocal of the dilution which with 3-agglutination units of the virus gives a two-plus agglutination pattern (maximal agglutination-pattern noted as four-plus). All sera freed from non-specific inhibitor.

sera of ferrets which were inoculated with garglings, sputum or autopsy material. The former procedure is the quicker one, and may even supply the answer when using amniotic fluids of sufficient titer originating from the first amniotic inoculation.

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ON THE ACTION OF SULFANILAMIDE

XII. Non competitive antagonists for Sulfonamides [1]).

by

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INTRODUCTION.

In a previous paper (6) we showed that the action of sulfanilamide (S.A.) against *E. coli* is antagonized non competitively by a series of five antagonists: methionine, xanthine, serine, thymine o r pteroylglutamic acid, and finally valine. This seems to indicate that increasing concentrations of S.A. interfere successively with the biosynthesis of these substances.

The present paper intends to show that derivatives of sulfanilamide inhibit the same set of enzyme systems.

As the previous communication was published elsewhere, a short discussion of the theory of non competitive inhibition is repeated here.

When the drug I inhibits the enzyme E, by competition with the substrate S for the enzyme-surface, an increase in the concentration (S) will antagonize the inhibition. A constant effect will be obtained by a constant ratio $\frac{(I)}{(S)}$ independent of the absolute value of the concentration (I). S is the competitive antagonist.

When such an enzyme system however is a part of a chain of enzyme reactions as in biosynthesis f.i. in bacterial growth, the addition of the endproduct P of the single step, catalyzed by E (and inhibited by I), will restore bacterial growth, though E remains inhibited. P is the non competitive antagonist. The concentration of P which restores growth is independent of the concentration of I.

[1]) I: Ann. de l'Inst. Pasteur **62**, 616, 1939. II: Antonie van Leeuwenhoek **7**, 25, 1941. III: Ibid. **7**, 77, 1941. IV: Ibid **7**, 153, 1941. V: Ibid. **7**, 161, 1941. VI: Ibid. **8**, 10, 1942. VII: Ibid. **8**, 86, 1942. VIII: Ibid. **8**, 139, 1942. IX: Ibid. **9**, 115, 1943. X: Ibid. **10**, 1, 1944—1945. XI: Arch. of Biochemistry **18**, 97, 1948.

The same reasoning holds when I is competing with the prosthetic group of the enzyme E for the surface of the apoferment. As in that case it is really the synthesis of the holoferment which is inhibited by I, the prosthetic group acts as a competitive antagonist. P however restores growth non competitively.

For the case that several enzymatic reactions, starting from the same substrate or catalyzed by the same prosthetic group, are inhibited by I, SHIVE (4,5) supposed that at increasing concentrations of I an increasing number of enzyme-reactions (E_1 to E_n) leading to the endproducts P_1 P_2 . . P_n is blocked. In low concentrations of I, the endproduct P_1 of the most sensitive reaction (E_1) will restore growth. In higher concentrations of I, P_1 becomes inactive because E_2, E_3 etc. are now also inhibited. But as the complex E_2I is more dissociated than E_1I (or less I is adsorbed on E_2 than on E_1 at a certain concentration of I) less S is necessary to restore growth in the presence of P_1. So on addition of P_1 the ratio (I/S) will increase.

P_2 will only be a non competitive antagonist unless P_1 is present and P_n will only show competitive antagonism if P_1, P_2 . . . P_{n-1} are present.

In the special case of sulfanilamide it was since long known that para-aminobenzoic acid (p.a.b.a.) was a competitive antagonist for practically all S.A. sensitive organisms. Since LAMPEN and JONES (3) found that pteroylglutamic acid was non competitively antagonizing S.A. in *Streptococcus faecalis* R and *Lactobacillus arabinosus*, this substance seemed to be the endproduct of the reaction blocked by S.A.

With other micro-organisms the mechanism seemed more complicated as pteroylglutamic acid was inactive and many other non competitive antagonists were found for various bacteria.

The confusing evidence on non competitive S.A. antagonists seemed to show with clarity only that S.A. directly or indirectly interfered with several metabolic reactions in most cases.

By the work of SHIVE (4) it was known that methionine and xanthine were non competitive S.A. antagonists for *E. coli*. Using SHIVE's method of inhibition analysis we were able to show (6) that for *E. coli* five non competitive sulfanilamide antagonists existed. Increasing concentrations of S.A. seem to inhibit the synthesis of methionine (m), xanthine (x), serine (s), thymine (t) and valine (v). Whether p.a.b.a. has the rôle of a substrate or a catalyst in these reactions we will not discuss here.

EXPERIMENTAL.

E. coli was grown in a synthetic medium containing NH_4Cl, glucose and salts. About 2×10^3 viable bacteria per ml were used as an inoculum. Growth was measured as turbidity in a Moll extinctometer. The sulfonamides studied were: sulfanilic acid (S), sulfanilamide (S.A.), sulfapyridine (S.P.), sulfapyrimidine (S.Pm) and sulfathiazol (S.T.). The antagonists were used in the optimal

concentrations established in the previous paper. For fuller particulars the latter should be consulted.

RESULTS.

Experiment I. The series of non competitive antagonists for sulfanilamide derivatives.

To establish, whether the same non competitive antagonists were active against other sulfonamides, *E. coli* was cultured in 10 ml tubes with high concentrations of sulfonamides, various concentrations of p.a.b.a. and constant concentrations of the non competitive antagonists. Growth was measured at 16, 24, 40, 48, 60, 72 and 96 hours.

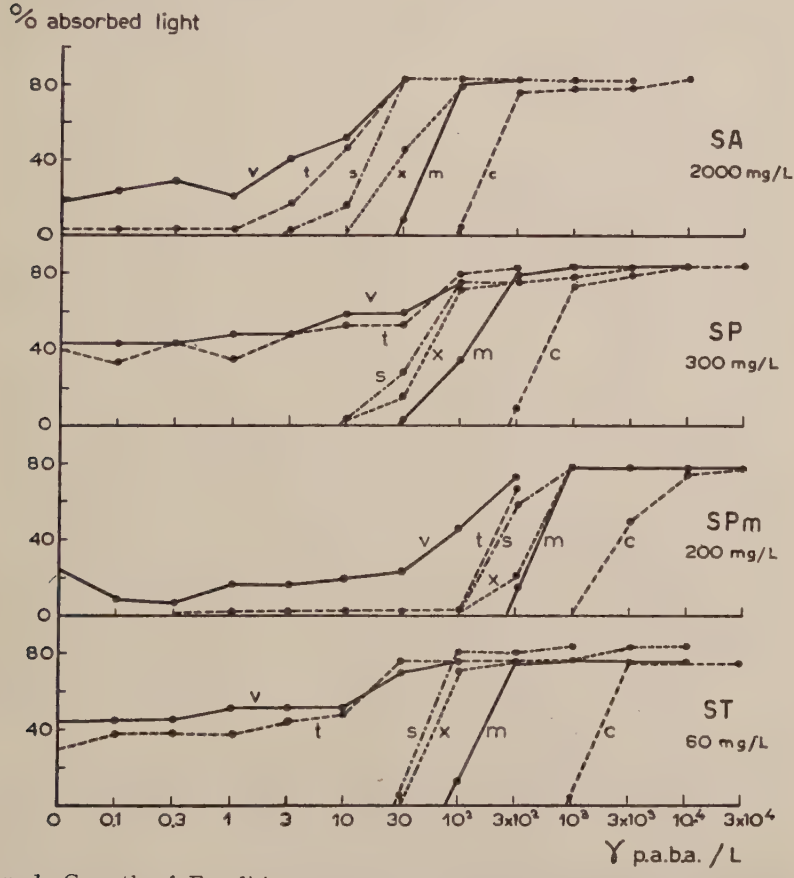

Fig. 1. Growth of *E. coli* in a synthetic medium with large concentrations of sulfonamides, varying concentrations of p.a.b.a. (abscissae) and optimal concentrations of the five non competitive antagonists.

Fig. 1 shows the resulting growth (turbidity) as a funtion of the p.a.b.a. concentration (logarithmic scale!) after forty hours (with time the curves shift to the left).

With 2000 mg/l sulfanilamide (Fig. 1 upper set of curves) no growth is obtained below 100 γ/l p.a.b.a.; however 300 γ/l p.a.b.a. restores growth completely (curve c). The addition of methionine (curve m) permits growth in 100 γ/l p.a.b.a., *i.e.* a shift of the antibacterial index $\frac{(\text{S.A.})}{(\text{p.a.b.a.})}$ of about three.

The further addition of xanthine (curve x) results in another shift of the antibacterial index. The series of tubes represented by curve x contain of course methionine and xanthine. Subsequent increases of the antibacterial index were obtained by the subsequent addition of serine (curve s), thymine (curve t) and valine (curve v), the latter mixture containing all five antagonists.

In the presence of all five antagonists (curve v) growth occurs without p.a.b.a. Indeed eight hours afterwards, at 48 hours, growth in these tubes was maximal.

In additional experiments it was shown that none of these antagonists was active unless the products lower in the series were present; f.i.: serine, thymine and valine are without effect in the absence of xanthine.

With 300 mg/l sulfapyridine (Fig. 1 second set) the same set of curves was obtained. With 200 mg/l sulfapyrimidine (third set) and with 60 mg/l sulfathiazol (lower set) the picture is the same. The mixtures with four (curves t) and with five (curves v) antagonists permit growth without p.a.b.a. A few hours later than at the time which is represented in fig. 1 all these cultures show maximal growth. The differentiation between t and v is indeed more evident at earlier times (f.i. at 24 hours). The results with sulfanilic acid are not shown in fig. 1 as the antibacterial activity is low and concentrations equal to 2000 mg/l S.A. could not be prepared. With the used concentration of 4800 mg/l maximal growth was obtained at 40 hours even with methionine alone. At 24 hours the results were however the same as with the other sulfonamides at 40 hours.

Though the p.a.b.a. concentrations at which growth occurs vary for each compound (the used sulfonamide concentrations being only approximately comparable), it is evident that the same non competitive antagonists are active for each of the drugs studied.

Sulfanilic acid and the three heterocyclic

sulfanilamid compounds seem to inhibit the same enzyme reactions as S.A. itself.

Experiment II. Comparision of the concentrations at which each enzyme system is inhibited by different sulfonamides.

Experiment I shows the shift of the antibacterial indexes and proves the existence of the five non competitive antagonists for each sulfonamide. To study the comparative sensitivity of each supposed enzyme system for the different sulfadrugs, another series of experiments was run. *E. coli* was cultured in the presence of varying concentrations of the sulfonamides (without p.a.b.a.) adding optimal concentrations of the five antagonists.

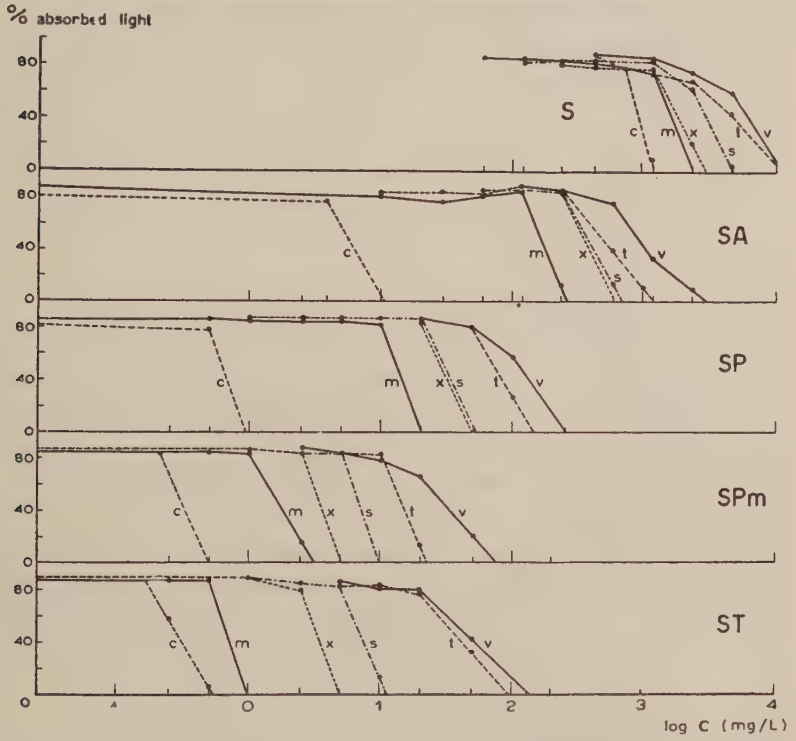

Fig. 2. Growth of *E. coli* in a synthetic medium with varying concentrations of five sulfonamides, in the presence of the five non competitive antagonists.

Fig. 2 is a typical example of this kind of experiment. Growth at 24 hours is plotted against the logarithm of the drug concentration.

Each of the sulfonamides inhibits growth at a certain concentration (curve c), on addition of each antagonist a stepwise increase in the sulfonamide concentration is necessary to obtain the same activity (curves m, x, s, t and v).

In the presence of all antagonists an increase of sulfonamide concentration of about hundredfold is necessary. Apart from the absolute concentration the picture is very much the same for the five sulfadrugs. (Not too much importance should be attached to minor differences in the relative position of the curves as the curves shift with time). This result was of course expected as it is generally agreed that various sulfonamides act by the same ,,intrinsic toxicity'' (2), differences in active concentrations being explained by physical properties of the molecule (1).

Still it is interesting that the sensitivity of the five enzyme

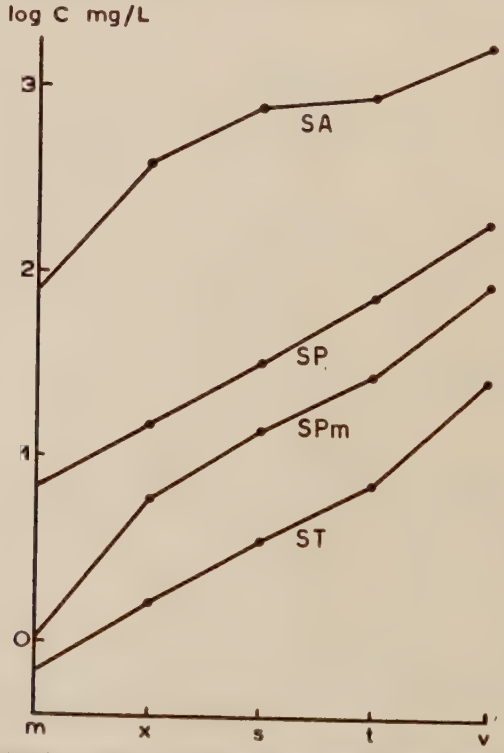

Fig. 3. Concentrations of four sulfonamides, which (presumably) inhibit the enzyme systems, synthesizing the five non competitive antagonists for 50 %. The enzyme systems are plotted along the abscis. m = methionine enzyme etc.

systems decreases along the series from methionine to valine in very much the same way for each drug. Fig. 3 representing a duplicate of Experiment II, illustrates this fact once more. In this graph the concentration necessary to obtain half maximal growth in the presence of each antagonist is plotted for each drug. The parallelism is evident. Attention may be drawn to the fact that the sulfanilamide concentration, which permits half maximal growth in the control is presumably the concentration which inhibits the methionine synthesizing enzyme at last partly. To obtain the exact concentrations which inhibits each system for 50 %, growth rates will have to be measured. We hope to report on them later.

The order of activity for the studied sulfonamides is: sulfanilic acid < sulfanilamide < sulfapyridine < sulfapyrimidine < sulfathiazol. The relative sensitivity of the five enzyme systems for each of these drugs is the same.

Summary.

In *E. coli*, sulfanilic acid, sulfanilamide, sulfapyridine, sulfapyrimidine and sulfathiazol are antagonized by the same series of non competitive antagonists, *viz.*, methionine, xanthine, serine, thymine and valine. This seems to indicate that the biosynthesis of these substances is successively inhibited by increasing concentrations of these sulfadrugs; the synthesis of methionine being inhibited first, that of valine only by excessive concentrations. Though the absolute concentrations vary with the drug the relative sensitivity of the five enzyme systems are very much the same. This again shows that the intrinsic toxicity of the sulfadrugs is the same.

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ON THE ACTION OF SULFANILAMIDE

XIII. Non competitive antagonists for Staphylococci [1]).

by

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(Received July 18, 1949).

In a previous paper (3, 4) we described a series of non competitive sulfanilamide-antagonists for *E. coli*. By the addition of these five antagonists full growth was obtained in a synthetic medium, in the presence of as much as 2000 mg/l sulfanilamide and without any para-aminobenzoic acid (p.a.b.a.). The five antagonists are: methionine(m), xanthine(x), serine(s), thymine(t) and valine(v) respectively; they probably represent the endproducts of five enzyme-reactions, which are consecutively inhibited by increasing concentrations of sulfanilamide (S.A.). For *Salmonella typhi-murium* the same series of antagonists was found active.

As the presence of non competitive antagonists in blood or tissue fluid will decrease the activity of sulfanilamides *in vivo*, it was important to study the non competitive antagonists for other bacteria.

This paper reports some of the results obtained with *Staphylococcus aureus*.

Experimental.

Three strains of *Staphylococcus aureus* were tested. One was our usual laboratory strain used as a coagulase positive standard (strain a). The other two were isolated from clinical material and were also coagulase positive (strain b and c).

The organisms were grown in a synthetic medium as described by Gladstone (1). The amino acids leucine, lysine and serine, however, were omitted. The final medium (double strenghth) contained:

[1]) XII: Antonie van Leeuwenhoek **15**, 129, 1949.

glucose	2.5 g	phenylalanine	40 mg
KH_2PO_4	4.5 g	histidine	40 mg
K_2HPO_4	0.5 g	arginine	70 mg
Fe $Am_2(SO_4)_2$	50 mg	aspartic acid	90 mg
$MgSO_4 . 7H_2O$	40 mg	glutamic acid	150 mg
glycine	25 mg	cystine	400 mg
alanine	50 mg	aneurine	4 mg
proline	70 mg	nicotinic acid amide	4 mg
oxyproline	80 mg	water	500 ml

The pH was adjusted to 7.5 and the medium sterilized by filtration through glassfilters. The medium was distributed in 5 ml portions to metal-capped extinctometer tubes. Sulfanilamide, p.a.b.a. or the antagonists methionine, xanthine, serine, thymine and valine were added up to a final volume of 10 ml.

The organisms were kept on meat infusion agar and subcultured for 24 h in the synthetic medium. One standard droplet (28 mg) of an appropriate dilution (usually 1 : 50) of this subculture was used as an inoculum for each 10 ml tube. All cultures were incubated at 37° C.

Growth was estimated in a Moll extinctometer as in previous experiments. The three strains of *Staphylococcus aureus* showed about equal sensitivity towards S.A.; growth was inhibited completely by 10 mg/l. The amount of p.a.b.a. necessary to restore growth in the presence of 2000 mg/l S.A. varied slightly for the three strains; 3 mg/l was necessary for strain a; 5—10 mg/l for strains b and c.

Results.

The essential feature about non competitive antagonists is the shift of the anti-bacterial index. In fact the addition of such an antagonist permits growth in a higher S.A. concentration when no p.a.b.a. is present. Again in the presence of large amounts of S.A. growth is obtained with smaller amounts of p.a.b.a. thus the anti-bacterial index $\frac{(S.A.)}{(p.a.b.a)}$ increases (2).

1°. Starting from the supposition that in the case of *Staphylococcus aureus* the same series of antagonists would exist as with *E.coli*, we tried whether methionine increased the anti-bacterial index, by growing *Staphylococcus aureus* (strain a) in the presence of 2000 mg of S.A. with varying concentrations of p.a.b.a. and 30 mg/l methionine. A concentration of p.a.b.a. of 9 mg/l was necessary to restore growth without methionine, whereas about 2 mg/l p.a.b.a. sufficed in the presence of this amino acid. The observed increase in anti-bacterial index seems to indicate that methionine is indeed the first non competitive antagonist for our

Staphylococcus strain. By repeating this kind of experiment with varying methionine concentrations it was established that 3 mg/l methionine was the smallest concentration which was completely effective. It is peculiar that this concentration is ten times smaller than with *E.coli*. Considering the activity of staphylococci in ,,assimilating''amino acids from the medium the methionine concentrations in the bacterial cell might be much more nearly equal for *E.coli* and *Staphylococcus*. However that might be, methionine seems active as the first non competitive antagonist.

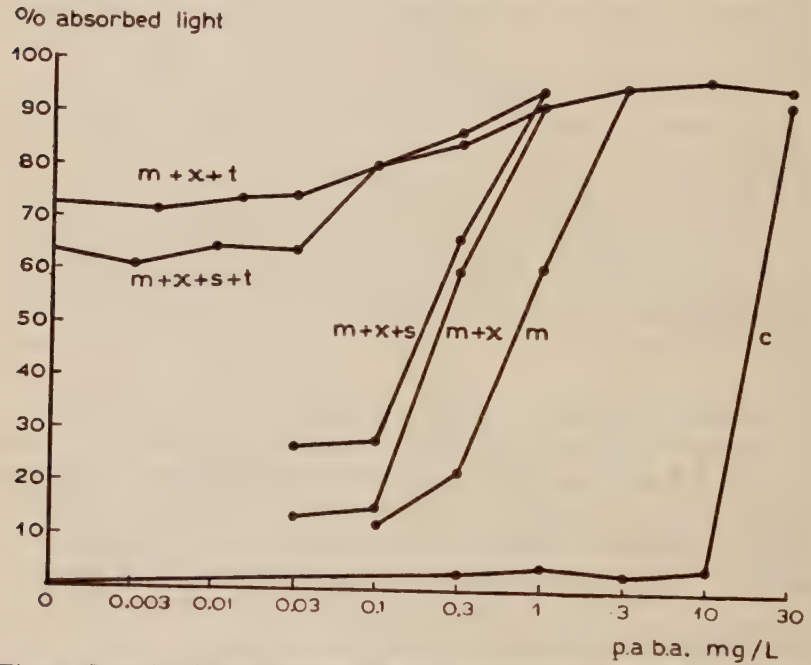

Fig. 1. Growth of *Staphylococcus aureus* in a synthetic medium with 2000 mg/l S.A., varying concentrations of p.a.b.a. (abscis) and optimal concentrations of the three non competitive antagonists.

2°. By culturing *E.coli* in a medium with 3 mg/l methionine, 2000 mg/l S.A. and various concentrations of p.a.b.a. it was established that xanthine acted as the second non competitive antagonist, as a further threefold increase in the anti-bacterial index was found. Without methionine, xanthine is of course inactive. The minimal active concentration was 3 mg/l (25 mg/l for *E.coli*). Guanine and hypoxanthine were less active than xanthine.

adenine was toxic. Xanthine acts as the second antagonist.

3°. For *E.coli* the third non competitive antagonist is serine. With concentrations from 2 to 50 mg/l serine, no further shift in the anti-bacterial index could be obtained with *Staphylococcus aureus* in media with optimal concentrations of methionine and xanthine. This seemed to prove that serine was not the end product of the third enzyme inhibited by S.A. Acid caseine hydrolysate and yeast extract were equally inactive.

4°. A further increase in the anti-bacterial index could however be obtained by the addition of thymine (i.e. the fourth antagonist in *E.coli*). The optimal concentration was 25 mg/l. Thymine acts as the third antagonist.

5°. A representative experiment is given in fig. 1 where growth (turbidity) is plotted against p.a.b.a. concentration. Staphylococci were grown in the described medium with a constant concentration of 2000 mg/l S.A., and varying concentrations of p.a.b.a. Six series were run, by adding optimal concentrations of the non competitive antagonists in different combinations.

curve c. control, growth is obtained between 10—30 mg/l p.a.b.a.

curve m. contains 3 mg/l methionine, full growth occurs with 3 mg/l p.a.b.a.; three to ten fold shift of the index $\frac{(\text{S.A.})}{(\text{p.a.b.a.})}$.

curve m+x. containing methionine and xanthine, further shift of index by xanthine (when only xanthine is added growth equals curve c, *i.e.* xanthine without methionine is inactive).

curve m+x+s. containing the former two and serine, the shift is insignificant and within the experimental error. This probably, though not conclusively, means that serine is not the third non competitive antagonist.

curve m+x+s+t. containing methionine, xanthine, serine, and thymine. The addition of thymine results in a very large shift of index; indeed growth is obtained here without any p.a.b.a. at all.

curve m+x+t. In this case serine is omitted. As the activity of thymine is not affected at all by this omission, serine definitely is not a non competitive antagonist. If it were, this curve should fall back to the xanthine curve, which happens indeed when this kind of experiment is performed with *E.coli*. By further experiments of this kind with various serine concentrations it was definitily

proved that serine has n o antagonistic properties for this strain of *Staphylococcus aureus*. In an analogous experiment it was found that pteroylglutamic acid even in concentrations of 1000γ/l is unable to replace thymine as it does in *E.coli*. However in this case 1000 mg/l pteroic acid replace thymine. Rhizopterine has no such activity.

As with thymine full growth is obtained in the presence of 1000 mg/l S.A. without any p.a.b.a., it seems that with methionine, xanthine and thymine the series of non competitive antagonists for staphylococci is complete.

6°. In the described experiments strain a was used throughout. Repetition of the experiments with strain b and c gave essentially the same results.

7°. In *E. coli* valine is a non competitive antagonist when m,x,s, and t are present. As with *Staphylococcus aureus* m,x, and t permit complete growth without p.a.b.a. in high S.A. concentrations, it seemed at first that valine played no such role with Staphylococci.

Whether valine is active at still higher S.A. concentrations could not be studied as these S.A. concentrations are no longer antagonized by p.a.b.a. thus entering the regions of unspecific S.A.-effects.

By using 100 mg/l sulphathiazol and various concentrations of p.a.b.a. a shift in anti-bacterial index between cultures with m,x,t, and cultures with m,x,t,v could be shown. Valine enabled the organism to grow without p.a.b.a. in this case.

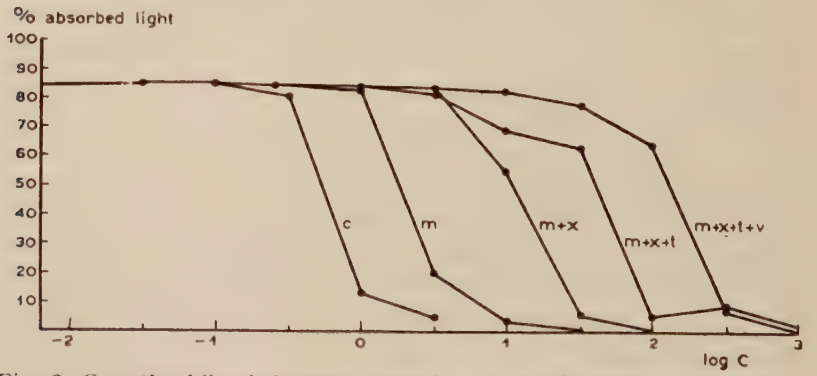

Fig. 2. Growth of *Staphylococcus aureus* in a synthetic medium with varying concentrations of sulfathiazol (abcsis) in the presence of four non competitive antagonists.

The existence of valine as the next antagonist is further illustrated by the experiment represented in fig. 2. Growth is plotted here against the logarithm of the sulfathiazol concentrations. Each curve represents a series of tubes into which the non competitive antagonists have been added. The addition of valine (curve m,x,t,v) evidently raises the effective sulfathiazol concentration about threefold. By the addition of all four antagonists the effective sulfathiazol concentration increases about 300 fold.

As the medium used here contains already ten amino acids the effect of valine is rather specific. This is further substantiated by the observation that acid caseine-hydrolysate is inactive.

Valine indeed seems to act as the fourth non competitive S.A.-antagonist for Staphylococci.

8°. For *E.coli* the series of non competitive antagonists is m,x, s,t,v, whereas for Staphylococci only m,x,t and v are necessary. As the described phenomena are so very much alike for Staphylococci and for *E.coli*, one wonders why serine is inactive in the former case.

It might be objected that in the used medium, with ten amino acids present, serine might also be inactive as a non competitive antagonist for *E.coli*.

By culturing *E.coli* in this medium it was easily established however, that serine is indeed a non competitive antagonist. In an experiment of the type of that in fig. 1 the addition of m,x,s,t,v permitted growth of *E.coli* without p.a.b.a.

The curve m,x,t,v omitting serine, nearly coincided with the curve m,x proving beyond doubt that serine is necessary. This fact once again shows the specificity of the non competitive antagonists.

In the amino acid medium the series of non competitive antagonists for *E.coli* is m,x,s,t,v, just as in ordinary media for *E.coli*.

Discussion.

The described experiments show that methionine, xanthine, thymine and valine form a series of non competitive antagonists for *Staphylococcus aureus*. According to the suppositions of inhibition analysis these substances seem to be the end products of four enzyme reactions in the bacterial cell which are inhibited by increasing S.A.

concentrations. The limitations of inhibition analysis and the validity of conclusions based on this method are of course fully realised. Keeping to the theory, however, the observed fact that in *S. aureus* serine is not included in the series of antagonists, seems to imply that the synthesis of serine is not inhibited by S.A. It is alluring to suppose that the serine synthesizing enzyme, which is present in *E.coli*, is absent in *S.aureus*; this would be in accord with the inability of most Staphylococci to synthesize their own amino acids from ammonia. *S.aureus* thus synthesizes its serine from other amino acids and this reaction is insensitive to S.A. Other possibilities are of course that *S.aureus* does not need or does not produce serine or even that it produces serine along the same pathway as *E.coli* but that the enzyme is S.A. insensitive. The first supposition seems more plausible however.

Regarding the inhibition of a valine synthesing enzyme by S.A. one has to assume that our *S.aureus* normally synthesizes valine from other amino acids or from ammonia.

The relative resistance of some staphylococcal infections to sulfadrugs might be explained (apart from transport difficulties of the drug to local processes) by the presence of p.a.b.a. and m,x,t,v in the host, especially in pus.

Summary.

With *Staphylococcus aureus* in a synthetic medium the action of 2000 mg/l sulfanilamide can be completely antagonized by a series of four non competitive antagonists *viz.*, methionine, xanthine, thymine and valine. Pteroylglutamic acid cannot replace thymine.

The implication is that the four enzyme systems leading to these substances are inhibited by increasing concentrations of sulfanilamide.

Serine is not a competitive antagonist for *S.aureus*, such as it is for *E.coli*. It seems that the serine synthesizing enzyme present in *E.coli* is absent in *S.aureus*.

References.

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NUCLEAR DIVISIONS IN THE FRUCTIFICATIONS OF SOME MYXOMYCETES AND A METHOD OF CULTURE TO OBTAIN FRUCTIFICATIONS

by

P. S. J. SCHURE

(Received July 20, 1949).

Introduction.

In 1928 for the first time Wilson and Cadman (15) reported the occurrence of two meiotic divisions in the fructification of the myxomycete *Reticularia Lycoperdon*. These divisions take place about fifteen hours after the emergence of the plasmodium from the wood in the sporogenic protoplasm. After meiosis has been completed the protoplasm is gradually cleaved into uni-nucleate portions, which become the spores. Schünemann (13) and Miss Cadman (1) who investigated *Didymium nigripes* var. *xanthopus* also found the meiotic divisions to occur before the formation of the spores. Compared with *Reticularia* there is a difference; in *Didymium xanthopus* meiosis and cleavage of the sporogenic protoplasm proceed simultaneously, the final cleavage into single spores taking place during the concluding stages of the second division.

In his study on the life-history of the Myxomycetes von Stosch (14) only found meiosis in one form out of four. This form resembles *Didymium nigripes* var. *eunigripes* as pictured in Lister's Monograph (11). Before the formation of the spores the nuclei of the sporogenic protoplasm divide simultaneously by mitosis; about twelve hours later the meiotic divisions occur in the maturing spores. In both meiotic divisions one of the daughter nuclei was seen to degenerate; in consequence of this the mature spore remains uni-nucleate.

In the other myxomycetes, *Didymium nigripes* var. *xanthopus*, *Didymium squamulosum* and *Physarum cinereum* von Stosch did not succeed in finding meiosis; instead of which he reports amitotic phenomena occurring in the spore. Having compared the pictures of Cadman with those of von Stosch I think that probably the *xanthopus* forms of both investigators are not identical. In Cadman's pictures four U-shaped chromosomes are seen in the metaphase of the first meiotic division, while in von Stosch's the metaphase of the division preceding spore-formation shows a larger number, about sixty of very minute chromosomes. Howard, Jahn and Miss Kränzlin observed like von Stosch only one division before the formation of the spores. Howard (7) in his paper on the life-history of *Physarum polycephalum* remarks: "Apparently only a single nuclear division requiring about thirty minutes takes place. Paired nuclei suggesting a second division have not been observed".

Jahn in his later papers (8, 9) is of the opinion that in *Badhamia* reduction is brought about in the single division preceding spore-formation. In an earlier work (1907) Jahn considered that the reduction process, initiated in the one division preceding spore-formation, in *Badhamia*, was completed in the first division of the spore.

Kränzlin (10) writes about *Arcyria cinerea*: "Die Karyokinese vor der Sporenbildung ist als heterotypische, die Schwärmerteilung als Reduktionsteilung anzusehen".

The above mentioned Mycomycetes all belong to the subclass of the *Endosporeae*. When I take the *xanthopus* form of Cadman as different from the *xanthopus* of von Stosch, investigations on meiosis have been made in nine forms of the endosporous Myxomycetes. In two cases, in *Reticularia Lycoperdon* and *Didymium xanthopus* "Cadman", the meiotic divisions were observed to precede the formation of the spores. In one case, of *Didymium eunigripes*, the two meiotic divisions were observed in the maturing spores. In two forms, *viz.*, in *Badhamia utricularis* and *Arcyria cinerea* there was only seen the first meiotic or the heterotype division, as indicated by a synaps and a diakinesis stage. In four forms, *Didymium xanthopus* "von Stosch", *Didymium squamulosum*, *Physarum cinereum* and *Physarum polycephalum* no meiotic divisions were found.

This paper deals with the nuclear behaviour before spore-formation in two other endosporous Myxomycetes, *Mucilago spongiosa*

(Leyss.) Morg. var. *solida* (Sturg.) Lister and *Physarum didermoides* (Pers.) Rost.

MATERIAL.

All fructifications were obtained from spore cultures. The spores of *Mucilago* came from one large fructification gathered by Mr. J. DAAMS in the vicinity of Eindhoven in October 1946 from the bark of a felled tree near a saw-mill. Part of this fructification was presented to me by Dr W. K. H. KARSTENS, to whom it was sent for determination. Later on also the spores of the sub-cultures were used to start cultures with. The genus *Mucilago* is described in LISTER's Monograph (11) as follows: "the sporangia are confluent to form an aethalium, otherwise the characters are those of the genus *Didymium*". In addition to the typical form of *M. spongiosa* two varieties are described. The var. *solida* is described as: "the aethalia pulvinate, compact, 4 to 5 cm diam., 1 to 2.5 cm thick, lime crystals small, often nodular; capillitium scanty, colourless, irregular; spores spinulose, 9 to 11 μ diam. The var. *solida* occurring usually on poplar bark, is a still more massive form (compared with the other variety), but it is connected with the typical form by intermediate gatherings".

The spores of the *Physarum* came from sporangia which had appeared on the soil of a diseased bulbplant kept in the room in May 1947, and of some subcultures. According to LISTER: "the sporangia measure about 0.8 mm high and 0.5 mm broad. The spores being very dark purple-brown, closely and minutely spinulose are from 10 to 13 μ in diameter. This form occurs on dead wood, leaves and old straw".

METHOD OF CULTURE.

The spores of *Mucilago* were sown on tapwater agar in a petridish. In twenty-four hours a number of the spores had germinated. Swarmcells provided with a flagellum were seen in the cultures at this time. In a two-day-old culture the number of swarmcells had increased and also amoeboid forms without a flagellum were seen. The latter are the amoeboid swarmcells. Both swarmcells and amoeboid swarmcells were observed to multiply by division. The amoeboid swarmcells were often seen in groups of two, three or four. In five-day-old cultures also larger bodies are present, the zygotes or young plasmodia. The process of fusion was not actually

seen, so it is uncertain whether it takes place in the swarmcell stage or in that of the amoeboid swarmcell. The agar cultures in which young plasmodia had already developed were supplied with a suspension of baker's yeast in tapwater. The yeast cells were observed within the amoeboid swarmcells and within the young plasmodia; so it appears that the baker's yeast serves as a food-substance for both. The plasmodia increase in size and their protoplasm soon is seen in streaming movement. By transferring them on fresh agar provided with a suspension of baker's yeast they grow rapidly and in a few days they spread over the whole surface of the agar plate. Frequent transferring of the plasmodium on fresh agar was necessary to keep the plasmodia in healthy condition. After a prolonged stay on the same culture medium, say for about a fortnight, the plasmodia deteriorated; sometimes encystment was observed. Neither by leaving them on the same culture medium nor by frequent transferring on fresh agar the plasmodia of this species were observed to fructificate. Cultures of *Didymium difforme* started at the same time and treated in the same way readily formed sporangia on the agar or on the glasswall of the petridish in about twenty days after the sowing of the spores. It seems, therefore, that the agar cultures are not suitable for *Mucilago* to arrive at the stage of fructification. A number of attempts has been made to get fructifications of this form by altering the culture conditions. The culturing of the plasmodia to obtain fructifications succeeded best when red earthenware flowerpots were used as a basis for the plasmodium instead of a petridish with agar. For this purpose flowerpots already used for the cultivation of plants in the garden were scrubbed clean with a brush, the hole in the bottom was stopped and the pots were kept in water until the pores were entirely filled. The pots were kept moist by placing them in basins containing tapwater which were covered with a glass plate.

Of a plasmodium grown on an agar plate pieces of at least one square centimetre were cut out together with the agar to which the plasmodium was attached. From these one piece was placed inside each flowerpot on the side wall. From the positive tropic reaction of the plasmodium against the earthenware it was clear that this medium was quite harmless. The plasmodia soon spread over the inside of the flowerpot and growth was abundant when a suspension of baker's yeast was given as food. The plasmodium everywhere leaves traces in the shape of a dark network. Usually

the reticulate plasmodium also grew and spread on the outside of the flowerpot, rather than cross over a place where it had already been. Under favourable conditions, *viz.*, a temperature from 20 to 25° C., adaequate moisture and a regular supply of food, the fructifications were formed after about fourteen days, counted from the transferring in the flowerpot. The fructifications nearly always appeared at the top of the flowerpot in a comparatively dry situation. The young fructifications were kept in a moist atmosphere for some days, before allowing them to dry up. When the process of drying up is too fast, the spores do not germinate.

The size of the fructifications grown in a culture never equalled that of the specimen gathered in the field. They were at most 1 cm in diameter and a 0.4 cm thick. By digging in the flowerpots in the garden in a shaded situation while the earth was kept moist with tapwater results were the same. The fructifications appeared at the same time as those kept in basins with tapwater. I did not have any opportunity to study the growth of the plasmodia when the tapwater was replaced by other salt solutions. It seems possible that the use of tapwater with its large amount of Ca-salts (about 40 mg Ca^{++} in a litre) is the cause of the short duration of the plasmodial stage and consequently of the smallness of the aethalia. As to the microscopic characters the size and markings on the spores were normal, though the spore coat of the cultivated material was darker and more spinulose than that of the wild form.

The culture of *Physarum* was likewise started with spores on agar, on which a yeast suspension was streaked to feed the plasmodia. Growth was good on this medium, but only rarely fructifications were formed. Sporangia formation in this form was better when the method of Camp (2) was followed. In this method instead of the agar basis filterpaper or gauze is used, and pulverised rolled oats are given as foodsubstance. Likewise sporangia are formed on earthenware. The plasmodium of *Physarum* took a longer period of growth than *Mucilago* when cultivated under similar circumstances as the latter. After about three weeks the sporangia were formed. Whereas the aethalia of *Mucilago* always appeared at the top of the flowerpot or on a dry place, the sporangia of *Physarum* were formed under more moist conditions. The plasmodium of *Physarum* before forming sporangia always moved downwards, usually on the outside of the wall, sometimes it spread over the surface of the water.

In the form of sporangia and in their crowded growth the cultured specimen of this form equalled the wild form from which the cultures were started.

CYTOLOGICAL TECHNIQUE.

To study the nuclear behaviour one of the acetic staining-fixation methods, which are described in DARLINGTON and LA COUR (4), was chosen. Bismarck brown used as a dye proved to be better for this material than carmine. With aceto-carmine the protoplasm is damaged and in this way the delicate nuclear structures burst. The composition of the fixing-staining fluid used is 0.5 % Bismarck brown (Merck) in 45 % acetic acid. Of the young fructifications samples were taken and smeared on coverslips. The smears were allowed to dry up by gentle heating and the coverslip was placed on a drop of acetic Bismarck brown on a slide. A slight pressure was applied under blotting paper and the edges of the coverslip were sealed up with a mixture of gelatine and acetic acid. Preparations kept for some hours proved to be better than those newly made. From these semi-permanent preparations a number was selected and made permanent by: 1° removing the cover slip from the slide with 10 % acetic acid, 2° rinsing the cover slip in 45 % acetic acid (twice), alcohol 96 % (twice), methylbenzoate, and 3° mounting in caedax.

Apart from the chromatine structures in the dividing nuclei the resting nuclei are less deeply stained with this method; in the protoplasm outside the nuclei some granula and ingested bodies stain deeply. Preparations made in the same way of amoeboid swarmcells and of young plasmodia, by making smears from agar cultures containing these stages, were less clear on account of the large amount of ingested bodies. The figures were drawn with the aid of a camera lucida using a Zeiss apochromatic objective 90/1,30 n.a. and a compensating ocular 15×.

THE CYTOLOGY AS SEEN IN THE STAINED PREPARATIONS.

The meiotic divisions in *Mucilago*. To study the nuclear behaviour in the sporogenic protoplasm five young fructifications were used. Samples were taken from these at regular intervals of time and stained with acetic Bismarck brown. The trials lasted from one and a half to twelve hours. An outline of these trials is given in table I, in which are taken up the

TABLE I.

Meiosis in the young aethalia of *Mucilago spongiosa* (Leyss.) Morg. var. *solida* (Sturg.) Lister.

number of the trial	date of trial 1948	time of day	number of samples	interval between samples	nuclear condition				formation of spore-wall
					resting stage	meiosis			
						prophase I	metaphase I	metaphase II telophase II	
1	Aug. 7/8	11.00 p.m.–0.30 a.m.	14	7 min.	11.00-11.15	11.15-	—	—	—
2	Sept. 21	8.30 a.m.– 3.45 p.m.	12	30 min.	8.30-11.30	11.30-1.00	1 p.m.-	—	1.50 p.m.
3	Sept. 28	8.30 a.m.–10.30 a.m.	13	10 min.	—	—	8.30-9.15	—	—
4	Sept. 28	7.00 a.m.– 6.45 p.m.	14	60 min.	—	—	—	7 a.m.	8 a.m.
5	Oct. 2/3	9.30 p.m.– 4.30 a.m.	16	30 min.	11.00-0.30	1.00-3.00	4. a.m.	4 a.m.	—

length of each trial, the number of samples, the interval between samplings, the nuclear condition and the appearance of the spore-wall. Trial 5 comprises the whole process of meiosis. The intervals between samplings amount to thirty minutes and as some of the preparations made contained degenerated material without nuclei, it is evident that stages of shorter duration may be easily overlooked. The further tests carried out help to amplify and to confirm the results obtained from trial 5. The following description of results has been derived mainly from trial 5. Where the results of other trials were used its number has been mentioned.

The contracting plasmodium had formed two white masses at 9.30 p.m., one measured about one cubic centimetre, the other was somewhat smaller; they were situated on the outside of the flowerpot

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EXPLANATION OF PLATE I.

All figures were drawn with the aid of a camera lucida and are magnified approximately × 1600. Figures 1 to 24 refer to *Mucilago*.

Fig. 1. Three resting nuclei in the protoplasm of the young fructification.
Fig. 2. Three nuclei in which some deeply stained granules are seen, showing the initiating of nuclear division in the fructification.
Figs. 3, 4. First prophase, leptotene.
Figs. 5, 6, 7. First prophase, stages of pairing of the chromosomes, probably the pachytene.
Fig. 8. Synapsis.
Fig. 9. First prophase, a nucleus reverting to a resting stage condition.
Fig. 10. First prophase, a later stage in pairing of the chromosomes.
Figs. 11, 12. First prophase, diakinesis?
Figs. 13, 14, 15. First metaphase, in side view, showing 10, 7 and 8 chromosomes.
Figs. 16, 17. First metaphase, polar views, with at least 8 chromosomes.
Fig. 18. First anaphase, early stage in side view.
Fig. 19. First anaphase, a later stage in side view; the boundary between the nucleus and the surrounding protoplasm has disappeared.
Fig. 20. A mass of protoplasm with both daughter nuclei of the first division in interphase.
Fig. 21. A mass of protoplasm with two nuclei, one in interphase, the other is dividing, probably in second prophase. Between the two nuclei are ingested bacteria.
Fig. 22. A mass of protoplasm with one nucleus in second metaphase.
Fig. 23. A mass of protoplasm with two nuclei; one in second anaphase, the other in second telophase.
Fig. 24. A larger oblong mass of protoplasm with two second telophases.

Plate I.

at some millimetres distance from each other. At twelve p.m. the smaller one had reached the bigger one and had completely fused with the latter. Up to 0.30 a.m. the nuclei remained in the resting stage. The resting nuclei (fig. 1) vary in size from 2.5 to 4 μ diameter, they are almost homogeneously stained, no nucleoli are seen in them nor can a definite nuclear membrane be observed with this method of staining. In most cases the nuclei are almost globular, often they are irregularly formed. Around the nucleus is a light area, which is practically empty, containing only some filaments with which the nucleus adheres to the surrounding protoplasm. This area has probably originated by shrinkage just within or outside of the boundary between the nucleus and the surrounding protoplasm. The nucleus together with the surrounding light area measure 5 to 6 μ in diameter. It is, therefore, also possible that the light area is part of the nucleus. In that case the stained body within would be the nucleolus or a karyosome. The living material does not show the nuclei.

The preparations made from 1 a.m. up to 3 a.m. show the nuclei in various stages belonging to a prophase. In trial 1 similar stages were found; as preparations in this trial were taken at seven minutes intervals, the sequence of the different stages in this series can be followed better. At the beginning of prophase a number of more deeply stained granules are seen in the homogeneously stained ground substance of the resting nucleus (fig. 2). Seven minutes later a number of nuclei was still seen to contain the deeply staining granules whilst other nuclei contained very tiny threads. It is presumed that this stage may be the leptotene (fig. 3). A very faintly stained spot is often seen in the nucleus at this stage (fig. 4). In this and in the following preparations there were also found stages with much thicker threads (figs. 5, 6 and 7). Apparently pairing of the chromosomes is taking place, probably these nuclei are in the pachytene. It is possible that the figs. 11 and 12 found in the same preparations represent the diakinesis condition. The later preparations of trial 1 do not show a metaphase stage. The nuclei were either in prophase or reverting to a resting condition. Fig. 9 shows such a nucleus reverting to a resting stage. This condition was noted also very often in the prophase of trial 5. According to DARLINGTON (3) this condition of partially or entirely reverting to a resting stage is inessential to meiosis; it occurs in some organisms in the pachytene or later in the first prophase.

Fig. 8 represents a synaps stage. This stage occurred in the later preparations of trial 1 and in the 3 a.m. preparation of trial 5; it was only rarely seen.

From the preparations in trial 5 made from 1 a.m. to 3 a.m. it is evident that a prophase of two hours' duration occurs in the young fructification. Because of this long duration and by reason of its nuclear structures, it may be concluded to be the first prophase of meiosis.

In the preparation made of a sample taken at 4. a.m. the metaphase, anaphase, and telophase of the first division have been found besides different stages belonging to a second division. The first metaphase was not so frequently seen in this series as in trial 3. At 3 a.m. it had not yet started. The preparation of 3.30 a.m. only contained degenerated material. At 4 a.m. the first metaphase for most of the nuclei had passed. For the study of this stage preparations of series 3 have also been consulted, in which the metaphase condition occurs almost exclusively. Fig. 13 represents a side view of the first metaphase. The width of the plate measures here 6 μ. The other metaphase conditions seen in side view measure only 4 to 4.5 μ (figs. 14 and 15). Apparently the structure in fig. 13 is smeared out so that the chromosome plate has become broader. Figs. 16 and 17 are drawings of first metaphase plates seen in polar view. The first anaphase was only observed sporadically. Fig. 18 shows an early first anaphase; the separation of the paired chromosomes is regular. A late first anaphase as represented in fig. 19 likewise occurs rarely. Apparently the first anaphase is only of very short duration.

After the first anaphase has been completed the protoplasm is divided up into more or less irregular, rounded masses varying in size. A number of these portions, measuring from 11 to 15 μ in diameter, will give rise to one spore.

The larger portions, some of which are oblong in shape, must still be cleaved up to form the spores. In some cases the beginning of such further cleavage was observed.

Fig. 20 represents a mass of protoplasm in which both daughter nuclei of the first division are present connected by a distinct spindle. The daughter nuclei are rounded in form and fairly deeply stained. In fig. 21 one of the daughter nuclei is seen in interphase condition, the other is dividing, probably the second prophase has been arrived at. In fig. 22 only one nucleus is visible in second

metaphase seen in side view. In fig. 23 two nuclei are present, one in second anaphase, the other in second telophase (side views). Fig. 24 is an oblong mass of protoplasm with two second telophase stages in it. Fig. 25 is a mass of protoplasm with five nuclei. The two below, more or less triangular in shape may be the daughter nuclei of the second division. The large and faintly stained one is probably a daughter nucleus of the first division which is still able to divide; the two deeply stained ones are probably degenerate nuclei. They are very deeply stained and seem to be surrounded by a vacuole. Fig. 26 is a mass of protoplasm in which only two daughter nuclei of the second division are seen. Fig. 27 shows a mass with two nuclei of the first division, one is still able to divide, the other is degenerate. An uncommon condition is shown in fig. 28, a small mass of protoplasm containing seven nuclei, four of which deeply stained, the other three weakly. The four deeply

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EXPLANATION OF PLATE II.

All figures were drawn with the aid of a camera lucida and are magnified × 1600. Figures 25 to 32 refer to *Mucilago* and figures 33 to 39 to *Physarum*.

Fig. 25. A large mass of protoplasm showing three nuclei of the first division, two of which are degenerate and two daughter nuclei of the second division in late telophase.

Fig. 26. A large mass of protoplasm with two daughter nuclei of the second division in late telophase.

Fig. 27. A mass of protoplasm with two nuclei, one able to divide, the other degenerate.

Fig. 28. A small mass of protoplasm with seven nuclei, four of which are degenerate.

Fig. 29. A mass of protoplasm with two nuclei surrounded by a vacuole.

Fig. 30. Masses of one-spore capacity with two deeply stained nuclei lying outside.

Fig. 31, 32. Young spores, showing beginning of wall-formation.

Fig. 33. A resting nucleus with the surrounding protoplasm of the young sporangium of *Physarum*. In the protoplasm deeply staining granules.

Fig. 34. A prophase.

Fig. 35. A metaphase.

Fig. 36. An anaphase with the surrounding protoplasm.

Fig. 37. First cleavage, two masses of protoplasm, in one a pair of telophase nuclei; in the other one nucleus, the second is seen in the protoplasm between the two portions.

Figs. 38, 39. Portions of one-spore capacity.

Plate II.

stained ones presumably are degenerate nuclei as in figs. 25 and 27. Later on such nuclei are surrounded by a vacuole and are digested as is shown in fig. 29. In a later preparation in which cleavage had proceeded further the deeply stained nuclei were also observed to lie outside the masses which become the spores (fig. 30). Trial 5 has ended here.

The formation of the spores was studied in trial 4. This trial shows some sporadic second metaphase and second telophase structures. Figs. 31 and 32 are from a preparation made thirty minutes later than the one drawn in fig. 30. A beginning of wall-formation occurs, which hinders the observation of the weakly stained nuclei. In fig. 31 one probably degenerate nucleus is visible.

The preparations of series 5 and 4 make evident that a second division of very short duration exists; this division occurring in the cleaved portions of the protoplasm, some of which are of one-spore capacity; the majority is larger.

As to the nuclei in the spore it was observed from the cleaved portions of one-spore capacity in which wall formation had not yet started, that a large number of spores is without a nucleus, in the others the number of nuclei may vary from one to four and occasionally even seven were counted. It is presumed that a part of these nuclei — those which stain deeply — is degenerate; sometimes they were seen lying in a vacuole or outside the mass of one-spore capacity. Conditions in the mature spore could not be investigated with the acetic Bismarck brown stain method.

In making germination tests of the wild form and of the cultivated specimens the germination percentage of the spores was found to be approximately 10 % for the first and 4 % and 20 % for two different cultivated specimens.

These numbers agree well with those reported by GILBERT (6) who made a comparative study of spore-germination in the Myxomycetes. He writes about *Mucilago*: "Germination of the thick-walled spores of the one fairly common species *M. spongiosa* first occurred after seven days and continued for about two weeks, the average percentage of different sowings being 10—15 %". As compared with *Reticularia* where germination takes place in very short time (thirty minutes to two hours) and the percentage of germination is usually 100 %, the spores of *Mucilago* do not germinate well. Apart from the external conditions influencing the process of germination GILBERT (5) distinguishes three causes affecting

germination: 1. The age of the spores; 2. the degree of maturation of the spores; 3. individual differences between species.

In the experiments made with material of the wild form, the percentage of germination did not seem to be much affected by a two years' preservation; and this material seemed to have matured under favourable conditions. I am of the opinion that the low germination percentage in this species must be attributed to the degenerating of a large number of nuclei during and after meiosis and to the irregular distribution of the nuclei over the spores.

The division before spore formation in *Physarum didermoïdes*.

In this species one division was found in the young sporangium occurring before cleavage of the protoplasm. A second division in the cleaved portions was not observed. Two trials were made, an outline of which is given in table II. The first trial on 15 August 1948 was started at 7.15 a.m. The sporangia had already been formed at this time. As the sporangia are small, the smear preparations were always made of a whole sporangium. It appeared that the sporangia from the same plasmodium are not in exactly the same stage; earlier stages might occur in later preparations.

From 7.15 a.m. to 1.20 p.m. the nuclei seen were in the same condition. They look like vesicles of 9 to 12 μ in diameter, in which a network of tiny and very faintly stained threads can be noted (fig. 33). Within them a faintly stained spot was sometimes observed. The surrounding protoplasm has taken on more stain and contains a number of deeply stained granules. As these nuclei differ from the resting nuclei seen in the young fructification of *Mucilago* and consequently their character of resting nuclei seemed doubtful, I compared them with the structures seen in preparations made from plasmodia of *Physarum*, which were in active feeding condition. In one preparation the nuclei appeared as more deeply stained globular or irregular bodies of 2.5 to 3.5 μ surrounded by a light area, together measuring 5.5 μ. In a preparation made of another plasmodium, which had been treated with colchicine similar faintly stained structures were seen lying in the vicinity of the more deeply stained nuclei. They measured only 5.5 to 6 μ. The occurrence of two types of nuclei was reported by Prowazek (12), von Stosch (14) and Howard (7). Prowazek noted in the plasmodium of *Physarum psittacinum* nuclei of two types. He

TABLE II.

Mitosis in the young sporangia of *Physarum didermoïdes* (Pers.) Rost.

number of the trial	date of trial 1948	time of day	number of samples	interval between samples	nuclear condition				
					resting stage	prophase	metaphase	anaphase	telophase (and cleavage)
1	Aug. 15	7.15 a.m.–3.05 p.m.	28	7 to 30 min.	7.15-1.20	1.40-1.47	1.40-1.56	2.07 p.m	2.15-2.50
2	Aug. 21	7.15 a.m.–5.40 p.m.	26	7 to 30 min.	7.15-5.00	5.05-5.15	5.05-5.15	—	5.40 p.m.

describes them as "helle succulente und fast gleichgrosze dunkle chromatinreiche Kerne". von Stosch found in old plasmodia and in young frucitifications of *Didymium xanthopus* a different type of nucleus than in young plasmodia. He considers that the type occurring in old plasmodia and in the fructifications, having a very small nucleolus is a „Hungertypus". Howard distinguished in the sporangium of *Physarum polycephalum* larger lightly stained nuclei and smaller densely stained ones. He believes the former to be in early prophase and the latter to be disintegrating. I am not certain whether the lightly stained nuclei are resting nuclei in a special condition or whether they are in early prophase. In the latter case this prophase condition would have a duration of 10 hours.

At 1.40 p.m. some prophase nuclei were seen (fig. 34), the greater part being in metaphase (fig. 35). At this stage the chromatic material formed a more or less compact plate like Howard described for *Physarum polycephalum*. The metaphase lasted till 2.07 p.m. Then the anaphase (fig. 36) and some telophase conditions were seen. At 2.15 p.m. the protoplasm had cleaved and formed portions of approximately 18 μ in diameter, in each of which a pair of telophase nuclei could be observed (fig. 37). Sometimes one of the daughter nuclei was seen lying in the cleaved portion and the other in the protoplasm between two portions. In few cases the mass of protoplasm contained three nuclei. On the whole, however, each mass was formed around a telophase mitotic figure. The same preparation contained areas in which cleavage had gone further and portions of 11 to 14 μ in diameter had been formed, which became the spores (figs. 38 and 39). Each portion contained one nucleus; only rarely portions with two nuclei giving rise to one spore were observed. The later preparations of this series showed the same stages or earlier ones. The second trial was started 21 August 1948 at 7.15 a.m. The nuclei remained in the same condition as in the first trial up to 5 p.m. At 5.05 p.m. in the same preparation the majority of the nuclei was in metaphase and only few were in prophase. At 5.15 p m. the nuclei were in the same condition. At 5.40 p m. the protoplasm had cleaved and formed portions of one-spore capacity, with one or two nuclei visible in them.

The study of these two series shows that the single mitosis occurring in the sporangia of *Physarum didermoïdes* takes place at least 10 hours after the formation of the sporangia. The nuclear divisions in the sporangium have a duration of 30 to 50 minutes.

The nuclear conditions in the maturing spores were not studied. This species differs from *Mucilago* in the type of nuclear division in the fructification and in the condition of the nuclei before these divisions start.

Discussion.

After comparing the results obtained in this investigation with the previous work on this subject it appears that:

In *Mucilago* the nuclear divisions in the fructification resemble those in *Reticularia Lycoperdon* and *Didymium xanthopus* of Cadman. *Mucilago* with the first or heterotype division in the uncleaved protoplasm and the second or homotype one in the cleaved portions takes an intermediate position between *Reticularia*, in which meiosis precedes cleavage and *Didymium xanthopus* in which meiosis and cleavage take place simultaneously. As regards the formation of the spores in *Mucilago* this process did not develop so regularly as in *Reticularia* or *Didymium xanthopus*. In *Reticularia* and *Didymium xanthopus* cleavage proceeds until the protoplasm has been divided up in uni-nucleate portions around which the spore coat is formed. In *Mucilago* after the final cleavage the protoplasmic portions, though approximately equal in size contain a varying number of nuclei, whilst a large number of the spores formed is without a nucleus.

As regards the viability of the daughter nuclei of the heterotype division and of the nuclei in the immature spore it was found that in *Mucilago* a large amount of these nuclei seemed to be degenerate. Degeneration of similar nuclei in *Reticularia* and *Didymium* is not reported in the literature. It seems probable that a relation exists between viability of the nuclei formed during meiosis and the percentage of germination of the spores.

Physarum didermoïdes resembles *Physarum polycephalum* and the forms studied by von Stosch respecting the one mitotic division before the formation of the spores. However, when the number of nuclei in the maturing spores is taken into account, there only exists a similarity with *Physarum polycephalum* for which Howard states that it has only a single resting nucleus, although rarely a young spore having two nuclei may be seen. Right after their formation the young spores in the forms studied by von Stosch often contain two nuclei, one of which is degenerate and is surrounded by a vacuole.

Summary.

1. Meiotic nuclear divisions have been found in the young fructification of *Mucilago spongiosa* (Leyss.) Morg. var. *solida* (Sturg.) Lister. The first meiotic division takes place in the uncleaved protoplasm; the second in the cleaved portions, which are partly of one-spore capacity. The immature spores vary in number of nuclei; a large number did not contain a nucleus.

2. The fructifications of *Mucilago* have been obtained in cultures started from spores. The method of culture is described. No attempts have been made at the purification of these cultures.

3. In *Physarum didermoïdes* (Pers.) Rost. only a single mitosis occurs in the young sporangium before the formation of the spores. The nuclei in the young sporangium are of another type than those in the plasmodium of the same species and in the fructification of *Mucilago*. The immature spores are uni-nucleate.

4. *Mucilago* appears to behave along the same line as *Reticularia Lycoperdon* and *Didymium xanthopus* (of CADMAN).

Physarum didermoïdes is in conformity with *Physarum polycephalum*.

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(From the Virus Laboratory of the University Medical Clinic, Leyden).

ANTIBODY RESPONSE AGAINST STRAINS OF INFLUENZA-A VIRUS IN FERRETS WITH BASIC IMMUNITY

by

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In the course of cross haemagglutination inhibition tests with ferret sera against different A-strains it appeared that the serological pattern of the strain A' 1265 (1948 U.S.A.) kindly sent to us by Dr R. M. TAYLOR (Rockefeller Institute for Medical Research, New York), differed considerably from all serologically analysed strains from epidemics of the 1933—1949 period. Apart from antibodies against its own strain, the antiserum also showed a high titer against the strains Shope-15 (swine-influenza), WS (1933 E), PR8 (1934 U.S.A.), A (1941 N) [2]), Barratt (1947 E) (A') and Rhodes (1947 U.S.A.) (A'). As until then we had been able to classify all the analysed A-strains into one of the ,,groups'' (,,Swine-virus''), (,,WS''), (PR8 + A (1941 N)), and A' [3]), the polyvalent serological pattern of A' 1265 was most uncommon. An explanation could be found, however, in the fact that the prae-infectioun ferret serum showed antibodies against the strains WS and PR8 (table 1). As both sera had been freed from non-specific inhibitor by treating them with enzyme from *Vibrio cholerae*, none

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[2]) Isolated in Holland during the 1941-epidemic

[3]) The first strains of this sub-group A' were isolated by ANDERSON and BURNET in Australia at the end of 1945 and the beginning of 1946. By American investigators the group is denoted as A-prime (A').

TABLE 1.

Haemagglutination inhibition test with pairs of ferret sera against influenza A stock strains and the homologous strains.

Pairs of Ferret serum	Strains								
	Lee (Infl. B)	Swine (15)	WS (1933 E)	PR8 (1934 U.S.A.)	A (1941 N)	Barratt (1947 E)	Rhodes (1947 U.S.A.)	A (Ned. I/49)	1265 (1948 U.S.A.)
1265 (1948 U.S.A.) (*a*)	<12/<12	<12/1024	**162**/13824	**42**/11946	<12/5376	<12/672	<12/5376	. . .	<12/864
1265 (1948 U.S.A.) (*b*)	<12/<12	<12/<12	<12/<12	<12/24	<12/198	<12/1380	<12/1610	. . .	<12/10240
A (Ned. I/49) (*a*)	<12/12<	<12/<12	<12/864	**198**/9557	**93**/11946	<12/336	<12/896	<12/512[1])	. . .
A (Ned. I/49) (*b*)		<12/<12	<12/<12	<12/<12	<12/<12	<12/320	<12/760	<12/93[1])	. . .

a = ferret serum with basic antibodies against two stock strains of influenza A.
b = ferret serum without basic antibodies.
The titer of the sera is expressed as the reciprocal of the dilution which with 3-agglutination units of the virus gives a two-plus agglutination pattern (maximal agglutination-pattern noted as four-plus). All sera freed from non-specific inhibitor.
[1]) Strain A (Ned. I/49) shows low titers against homologous and heterologous antisera.

of the titers could be explained by their being due to this non-specific inhibition. It might conceivably be that inadequate inactivation of the cholera enzyme after interaction with the immune serum could have caused the high titer with all the strains, but the same result was obtained when the test was repeated with the same sera which after the treatment with cholera enzyme had been heated for 1½ hour at exactly 56° C. We must therefore assume that owing to basic antibodies the ferret had a prae-infectioun titer against the sub-groups (WS) and (PR8), and that after immunization with the strain A' 1265, an increase in titer had taken place against these two and the swine virus, as well as against the sub-group A'. From this it could be concluded with some probability that the strain belonged to the A'-sub-group. In fact a fresh ferret serum against the strain A 1265 yielded the serological pattern of an A'-strain (table 1), as already established by TAYLOR (6).

Curiously enough, we found at the same time a second example of a polyvalent antiserum originating from a ferret which was inoculated with a strain isolated in Holland during the 1949-epidemic, and containing basic antibodies, now demonstrable against the strains PR8 and A' (Ned I/1949). In this case however, it failed to show a rise against the swine influenza virus. A new antiserum against this strain yielded the serological pattern of other 1949-strains isolated in Holland (table 1), which is almost similar to that of the strains isolated in Europe and America in 1947 (A'). Both ferrets proved to have been derived from the same dealer, and they were older animals from a zoological garden, which we were compelled to buy, as our stock of ferrets had greatly dwindled by the beginning of 1949. FRANCIS and MAGILL (4) also mentioned the occurrence of ferrets with basic antibodies, which could not be traced to a known laboratory infection. It therefore appears that a ferret which has basic antibodies against the group (WS) and/or (PR8), and is infected with a strain of the A'-group, also develops a rise in antibodies against both the former groups. TAYLOR (6) recently described the same phenomenon. In the above-mentioned tests it remains unexplained why in the first case a rise against the Shope-virus occurred, failing, however in the second case.

It is well known, that similar observations can be made with sera from patients who have suffered from influenza (STUART-HARRIS *et al.* (1), BODILY and EATON (2), EATON and PEARSON (3), HORSFALL and RICKARD (5), TAYLOR and DREGUSS (7)).

TABLE 2.

Haemagglutination inhibition tests with human serum pairs against representatives of four A sub groups.

Pairs of Patient's Sera	Strains				
	Heer (1949 N) or Hess (1949 N)	Rhodes (1947 E)	PR8 (1934 U.S.A.)	WS (1933 E)	Shope (Swine)
Polderv (1949 N)	<12/2560	<12/2688	<12/47	... /<12 [1])	...
v. Zwa (1949 N)	15/640	...	<12/<12	<12/<12	<12/<12
van Hoof (1949 N)	<12/373	<12/1380	42/2688	...	...
Knof (1949 N)	<12/192	...	48/192	<12/126	<12/<12
Hofs (1949 N)	<12/768	...	160/3072	<12/2560	<12/3413
Mas (1949 N) [2])	<12/192-768	...	<12/168-597	<12/373-1344	84/168-224
Keyzer (1949 N)	<12/288	<12/2560	18/1610	28/448	106/2048

The titer of the sera is expressed as the reciprocal of the dilution which with 3 agglutination units of the virus gives a two-plus agglutination pattern (maximal agglutination pattern noted as four plus). All sera freed from non-specific inhibitor. All serum controls did not show agglutination of chicken cells.

[1]) Acute serum no more available.
[2]) Two post infectioun sera available.

If in human sera the non-specific inhibitor is eliminated from the serum pairs, it is possible, using the haemagglutination-inhibition test, to obtain a general idea of the existence of basic antibodies against one or more groups. It must be remembered however, that it is not possible to demonstrate small remnants of basic antibodies which would only be manifest in undiluted serum. Moreover the detection of the presence of basic antibodies may be hampered by non-specific agglutination of red cells in the lowest dilutions of the serum, and sometimes, perhaps, also by a final remnant of non-specific inhibitor. Also the first serum of the patient must date from the first days of the illness. In table 2 some examples are given of the rise of antibodies in sera from adults, suffering from influenza in the winter of 1949. This table shows how irregular rises in titer in human sera against different representatives of the sub-groups of influenza-A may prove to be. It is striking that the prae-infectioun titer against the 1949- and

1947-strains are with one exception less than 12. On two occasions (Polderv. and v.Zwa.) it is probable that there are no basic antibodies against two or three heterologous A-groups (PR8, WS and Shope-15). In both cases the post-infectioun rise against these strains is slight or altogether absent. In all other cases there is a basic titer against one or more heterologous A-strains with a post-infectioun rise against these strains. It is not possible to find a definite rule or any regularity in the post-infectioun patterns that were obtained. As amongst 77 serum-pairs (adults) showing a definite rise in antibodies, 55 proved to have no basic antibodies against A'-strains in the acute serum, it is very probable that the 1949-influenza-epidemic found a mainly clear field among the population of Holland.

Summary.

In Holland ferrets are offered for sale with basic antibodies against one or more sub-groups of strains of influenza-A virus. With the haemagglutination inhibition test low basic antibody titers can only be detected by neutralising the non-specific inhibitor in the ferret sera. When such a ferret is infected with a strain of a heterologous sub-group, a considerable rise in antibodies is obtained against the homologous and heterologous sub-groups. It is very probable that the same holds for human beings. Some examples are given of haemagglutination-inhibition tests with serum pairs from adults who suffered from influenza in the epidemic of the winter of 1949 in Holland from which the non-specific inhibitor had been eliminated.

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HAEMAGGLUTINATION DURCH POCKENVIRUS

7. Die Brauchbarkeit von Vaccine-Virus verschiedener Herkunft für die Haemagglutination [1])

von

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Nachdem sich in der Praxis des Instituts Pasteur die Karbouwenpulpa für die Haemagglutination ausserordentlich gut bewährt hatte, war es von Interesse auch Pulpa anderer Herkunft auf ihre Eignung dafür zu untersuchen. Kälbervaccine hatte sich schon in den Versuchen von NAGLER (6) als schlecht wirksam erwiesen, und BURNET und STONE (1, 7) hatten später in solcher einen Inhibitor nachgewiesen, dem sie Antikörper-Natur zusprachen. Die gleichen Autoren hatten auch in der Kaninchenhaut 3 Tage nach der Infektion mit Vaccine Haemagglutinine nachgewiesen und auch hier das schnelle Auftreten von hemmenden Stoffen gesehen. Ausgezeichnet eignet sich nach NAGLER und BURNET und STONE (1) Chorionallantois-Material, auch CHU (2) benutzte dieses Material für seine Versuche.

In der nachfolgenden Mitteilung wird über Versuche berichtet, Virus von der Haut vom Kalb, erwachsenen Kuh, Meerschweinchen, Kaninchen und Affe und ausserdem Testes-Virus vom Kaninchen und vom Meerschweinchen für die Haemagglutination zu benutzen. Ferner wurde die spezifische Hemmung der Haemagglutination durch Variolaserum bei der Benutzung dieser Viruspraeparate untersucht. Anhangsweise werden einige Befunde über Hemmungsstoffe im unwirksamer Kalbslymphe und wirksamem Kaninchen-Testes-Virus mitgeteilt.

Methodik.

Die Pulpa vom Kalb und von der Kuh (beide von indiascher Rasse) wurde 3 Tage nach der Hautimpfung geerntet, die Pulpa

[1]) Mitteilung 6: Antonie van Leeuwenhoek **15**, 97, 1949.

vom Kaninchen, Meerschweinchen und Affen (*Macaca irus mordax*) 4-5 Tage nach Infektion. Das Testes-Virus vom Kaninchen und Meerschweinchen wurde 3-4-5 Tage nach erfolgter intratestikulärer Infektion gewonnen. Gehirn-Virus vom Kaninchen war 6-7-8 Tage nach intracerebraler Infektion mit Virus von Testes-Passagen entnommen.

Das Virusmaterial wurde im Mörser mit physiologischer Kochsalzlösung zu einer 10%igen Suspension verrieben und bei 2000 Umdrehungen 10 Minuten lang zentrifugiert. Die überstehende Flüssigkeit wurde als Verdünnung 1/10 angesehen. Fallende Verdünnungen hiervon wurden in der Menge von 0,25 ml in Röhrchen von ca. 1 cm im Durchmesser mit der gleichen Menge einer 1%igen Hühnerblutsuspension versetzt. Die Haemagglutination wurde nach ungefähr 3 Stunden abgelesen. Die Versuche über Hemmung mit spezifischem Antiserum wurden in gleicher Weise durchgeführt, wie COLLIER (4) angegeben hatte.

Alle Impfungen wurden mit dem Karbouwen-Vaccinestamm des Instituts Pasteur angestellt, der von menschlicher Variola abstammt und via Affen und Kaninchen an das Kalb und den Karbouw angepasst worden war. Seine Fortimpfung erfolgt stets im Cyclus Kaninchen-Kalb-Karbouw.

Ergebnisse.

In der nachfolgenden Tabelle 1 sind die Versuche mit den verschiedenen Pulpen und mit Testes-Virus zusammengestellt. Da die Karbouwenpulpa bei Aufbewahren im Eisschrank in der Regel viele Monate lang unverändert bleibt und im getrockneten Zustand selbst nach 18 Jahren noch deutliche Haemagglutination ergibt, wurde bei den Pulpen von anderen Tieren der Einfluss des Aufbewahrens — wenigstens für kurze Zeit — berücksichtigt.

Zwei indische Kälber wiesen am Tage der Ernte einen Haemagglutinationstiter von 1/160 bzw. 1/320 auf, am folgenden Tage nur noch von 1/80 bzw. 1/20 und am Tage danach war in der Endverdünnung von 1/20 bei beiden keine Haemagglutination mehr zu sehen. Verschiedene andere Kälber wiesen auch bei Beginn keinerlei Haemagglutinine auf. Die Entwicklung der Haemagglutinin-Titer von 2 anderen Kälbern findet sich in Tabelle 2 zusammengestellt. Eines der Tiere zeigte am 71. Tage einmal einen geringen Titer. Das andere Tier nimmt unter allen untersuchten Kälbern insofern

Tabelle 1.

Schwankungen des Haemagglutinationstiters nach Aufenthalt im Eisschrank bei ± 4° C.

Virus von:	Entwicklung des Virus in Tier in Tagen:	Haemagglutination nach Aufenthalt bei ± 4° C.					
		direct	1 Tag	2 Tage	3 Tage	4-5 Tage	6-8 Tage
Indiasches Kalb 1	3 Tage	1/160	1/80	0			
,, ,, 2	3 Tage	1/320	1/20	0			
,, Kuh 1	3 Tage		1/640		1/20	>1/20	1/20
,, ,, 2	3 Tage		1/320		1/40	>1/20	>1/20
,, ,, 3	3 Tage		1/160		1/20	>1/20	>1/20
,, ,, 4	3 Tage		1/320		1/20	>1/20	>1/20
,, ,, 5	3 Tage	1/320	1/40		1/40		
,, ,, 6	3 Tage	1/80	1/40		>1/20		
,, ,, 7	3 Tage	1/320	1/80		1/80		
,, ,, 8	3 Tage	1/1280	1/40		1/40		
,, ,, 9	3 Tage	1/80	1/40		1/40		
,, ,, 10	3 Tage	1/40	1/20		>1/20		
Cavia-Haut a	4 Tage	1/2560				>1/20	
,, b	4 Tage	1/800	1/640				1/480
,, c	5 Tage	1/1280		1/240			
Affen-Haut a	4 Tage (*)	1/1280	1/640				
,, b	5 Tage						>1/20
,, c	5 Tage	1/640	1/640		1/320		
Kaninchentestes:							
I. Passage a	3 Tage	1/100	1/200		1/80		1/240
b	4 Tage	1/800		1/1280			1/1920
II. Passage a	5 Tage	1/160	>1/20	>1/20			
b	5 Tage	1/160	>1/20	>1/20			
III. Passage	4 Tage	1/800	1/960				
VII. Passage a	4 Tage	1/2560	1/480				
b	4 Tage	1/160	1/120				
VIII. Passage	4 Tage	1/1280	1/320				
Caviatestes:							
I. Passage a	3 Tage	1/100	1/20		>1/20		
b	4 Tage	1/200		>1/20			>1/120
II. Passage a	5 Tage	1/160	>1/20	>1/20			
b	5 Tage	1/160	1/960	>1/120			

(*) nach 6 Wochen in Glycerin = 1/80.

eine Sonderstellung ein, als es die ganze Versuchszeit durch agglutinierte, allerdings schwankte der Titer einigermassen.

Tabelle 2.

Schwankungen des Titers der Haemagglutination bei 2 Kälbern Indiascher Rinderrasse.

Aufenthalt bei + 4° C.	Titer der Haemagglutination:	
	Kalb A	Kalb B
Vor Glycerinzugabe		1/1280
Nach Glycerinzugabe		
nach 1 Tag	>1/20	1/400
3 Tagen		1/2560
4 „		1/800
5 „		1/400
13 „	>1/20	1/40
20 „		1/320
26 „	>1/20	1/160
32 „	>1/20	1/60
34 „	>1/20	1/240
71 „	1/40	1/320

Die Pulpa der indiaschen Kühe erwies sich am Tage der Ernte und am folgenden Tage als agglutininhaltig, später verlor sich die agglutinierende Eigenschaft ziemlich schnell.

Ein Rückgang des agglutinierenden Vermögens zeigte sich auch bei der Meerschweinchenpulpa und der Affenpulpa. Die Affenpulpa wurde nach 24 Stunden Aufenthalt im Eisschrank zu Glycerinvaccine verarbeitet und erwies sich als solche nach 6 Wochen noch als aktiv und agglutinierte bis zur Verdünnung von 1 80. Eine andere gefroren aufbewahrte Affenpulpa war am 5. Tage unwirksam.

Ein starker Rückgang im Titer zeigte sich auch bei den verschiedenen Versuchen mit Testes-Virus vom Kaninchen und Meerschweinchen.

Fast jedes untersuchte virushaltige Material von den verschiedenen Versuchstieren ging also beim Aufbewahren im Eisschrank an Wirksamkeit mehr oder weniger schnell zurück, ganz im Gegensatz zur Karbouwenpulpa, die fast stets monatelang unverändert blieb.

Interessant ist weiterhin der Nachweis von Haemagglutininen

Tabelle 3.

Titer der Haemagglutination durch Testissuspension von Kaninchen nach verschiedenen Passagen.

Testes-Passage:	Tier No.:	Dauer der Passage in Tagen:	Titer der Haemagglutination direct:
1	1	3	1/100
	2	3	1/100
	3	3	1/1280
	4	4	1/800
2	1	3	1/320
	2	5	1/160
	3	5	1/160
3	1	3	1/1280
	2	4	>1/20
	3	4	1/800
4	1	4	1/640
5	1	4	>1/20
	2	4	1/640
	3	4	1/1280
6	1	6	>1/20
	2	6	>1/20
7	1	4	1/160
	2	4	1/2560
8	1	4	1/1280
	2	4	1/1280
9	1	4	1/40
	2	4	1/2560
10	1	5	>1/20
	2	5	>1/20
12	1	4	1/80
	2	4	1/320
13	1	4	1/160
	2	4	1/320
14	1	4	1/640
	2	4	1/640
15	1	4	1/320
	2	4	1/320
19	1	5	1/640
20	1	5	1/2560
Gehirnpassagen			
1	1	7	1/640
2	1	6	1/2560
3	1	8	1/320

in den Kaninchentestes. Hier erwies sich, dass im Verlauf der aufeinanderfolgenden Passagen der Haemagglutinationstiter nicht gleichmässig blieb, vielmehr zeigten sich starke Schwankungen, die sogar soweit gingen, dass einzelne Passagen bei einem Tier deutliche Haemagglutination ergaben, beim andren Tier aber nicht. Diese Versuche sind in der Tabelle 3 zusammengestellt. Auch drei Gehirnpassagen wurden untersucht und zeigten gleichfalls Schwankung im Haemagglutinationstiter.

Besonders wichtig war aber der Nachweis, dass die Haemagglutination durch die verschiedenen virushaltigen Pulpen und Testes-Virus durch spezifisches Antiserum in analoger Weise gehemmt wurde, wie die Haemagglutination durch Karbouwenpulpa. In Tabelle 4 sind Versuche zusammengestellt, in denen Karbouwenpulpa und Kaninchen-Testes-Virus in 1-2-4- und 8-fachem Multiplum der eben noch agglutinierenden Dosis mit fallenden Verdünnungen von 7 verschiedenen Seren von Variola-Patiënten vorbehandelt wurden. Es ist hier der Endtiter des Serums angegeben, wobei komplette Hemmung der Haemagglutination erzielt wurde. Abgesehen von kleinen Differenzen hemmten die 7 Seren die Agglutination sowohl durch Karbouwenvirus als auch durch Kaninchen-Testes-Virus gleichmässig gut. Also letzteres ist ebenfalls gut brauchbar zur Feststellung der neutralisierenden Serumwirkung.

In der Tabelle 5 (Seite 174) sind weiterhin noch analoge Versuche wiedergegeben, bei denen Serum von Variola-Patienten gegen Pulpen verschiedener Tierarten untersucht wurden. Auch hierbei zeigte sich, dass die Haemagglutination durch frische Pulpa vom Affen, Kaninchen und Meerschweinchen mehr oder weniger in gleichem Masse wie Karbouwenpulpa durch Variolaserum gehemmt wurde.

Es fragte sich, ob das häufige Unvermögen von Kälberlymphe, Hühnererythrozyten zu agglutinieren, nur darauf zurückzuführen war, dass Hemmungsstoffe darin vorhanden waren, oder ob die Haemagglutinine überhaupt fehlten.

Karbouwenvirus wurde in den Verdünnungen von 1/300, 1/600 und 1/1200 mit verschiedenen Konzentrationen von nicht agglutinierendem Kälbervirus in nativem Zustand und nach 30 Minuten Erwärmen auf 56°, 63° und 80° C. versetzt und 1 Stunde bei 37° C. gebunden. Als Kontrolle wurden die verschiedenen Kälbervirusverdünnungen ohne Karbouwenvirus mit physiologischer Kochsalzlösung versetzt. Hierauf wurde Blut beigegeben und nach ca.

Vergleich der Hemmung der Haemagglutination durch Karbouwen Pulpa bzw. Kaninchen-Testes-Virus durch verschiedene Variolaseren.

Virusmaterial	Multiplum	Variolasera						
		1	2	3	4	5	6	7
Karbouwen Pulpa	1×	1/2560	1/2560	1/640	1/640	1/5120	1/12800	1/1600
	2×	1/2560	1/2560	1/160	1/160	1/1280	1/3200	1/400
	4×	1/1280	1/1280	1/160	1/160	1/320	1/800	1/200
	8×	1/640	1/640	1/40	1/40	1/320		
Kaninchen Testes	1×	1/5120	1/2560	1/640	1/320	1/5120	1/12800	1/800
	2×	1/2560	1/2560	1/320	1/160	1/5120	1/1600	1/200
	4×	1/1280	1/1280	1/80	1/40	1/2560	1/1600	1/100
	8×	1/640	1/640	1/40	1/20	1/1280		

Tabelle 6.

Hemmungskörper im unwirksamen Kälbervirus.

Karbouwenvirus-Concentration:	Kontrolle. Karbouwenvirus:	Vorbehandlung des Karbouwenvirus mit Kälbervirus:															
		Nativ				30′56° C.				30′63° C.				30′80° C.			
		1/30	1/60	1/120	1/240	1/30	1/60	1/120	1/240	1/30	1/60	1/120	1/240	1/30	1/60	1/120	1/240
—	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1/300	+	0	0	(+)	+	0	0	(+)	+	0	0	(+)	+	(+)	(+)	+	+
1/600	+	0	0	0	0	0	0	0	(+)	0	0	0	(+)	0	0	(+)	(+)
1/1200	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1/2400	0																

Tabelle 5.

Vergleich der Hemmung der Haemagglutination durch verschiedene Pulpas durch Variolaseren.

Virusmaterial	Multiplum	Variolasera:	
		Serum 1	*Serum 2*
Karbouwen Pulpa	1×	1/12800	1/12800
	2×	1/6400	1/6400
	4×	1/800	1/1600
Affen Pulpa	1×	1/12800	1/12800
	2×	1/800	1/3200
	4×	1/800	1/800
		Serum 3	
Karbouwen Pulpa	1×	1/6400	
	2×	1/3200	
	4×	1/400	
	8×	>1/400	
Kaninchen Pulpa	1×	1/6400	
	2×	1/6400	
	4×	1/3200	
	8×	1/400	
		Serum 4	
Karbouwen Pulpa	1×	1/5120	
	2×	1/1280	
	4×	1/320	
	8×	1/320	
Cavia Pulpa	1×	1/2560	
	2×	1/1280	
	4×	1/640	
	8×	1/160	

3 Stunden abgelesen. Der Versuche ist in Tabelle 6 (Seite 173) zusammengestellt.

Es zeigt sich, dass die Kälberlymphe in keinem Fall Hühnerblut agglutinierte, also unwirksam blieb. Die native und die auf 56° und 63° C. erwärmte Lymphe hemmte die vierfache Karbouwendosis gleichmässig stark, die doppelte Karbouwen-Virusdosis wurde durch native Kälberlymphe bis zur Verdünnung von 1/240 gehemmt, aber die auf 56° und 63° C. erwärmte Lymphe wirkte hier

schon etwas schwächer und hemmte bei 1/240 nicht mehr. Noch mehr abgeschwächt war die Hemmungswirkung nach Erwärmung auf 80° C., aber sie war doch noch deutlich vorhanden, also einigermassen thermostabil. Trotzdem die Hemmungsstoffe vermindert waren, zeigte sich durch die Kälberlymphe keinerlei Haemagglutination, sodass man daraus den Schluss ziehen kan, dass das fehlende Agglutinierungsvermögen der Kälberpulpa nicht auf Neutralisierung des Virus durch vorhandene Hemmungsstoffe zurückzuführen ist, sondern auf andere Faktoren, die wir noch nicht kennen.

Hemmende Stoffe finden sich nicht nur in nicht-agglutinierenden Viruspraeparaten, sondern auch in solchen, die deutlich Haemagglutination ergeben. Dies zeigt sich beispielsweise bei Kaninchen-Testes-Virus, dessen Haemagglutinine im Gegensatz zum Karbouwenvirus thermostabil sind. Drei verschiedene Konzentrationen Karbouwenvirus wurden mit fallenden Verdünnungen von

Tabelle 7.

Hemmungsstoffe im Kaninchen-Testes-Virus.

Karbouwen-virus-Concentration:	Kontrolle. Karbouwen-virus:	Vorbehandlung des Karbouwenvirus mit Kaninchen-Testes-Virus:					
		Vorbe-handlung:	1/30	1/60	1/120	1/240	1/480
—	—	Nativ	+	+	(+)	(+)	0
1/300	+	,,	+	+	+	+	—
1/600	+	,,	+	+	(+)	(+)	(+)
1/1200	(+)	,,	+	+	(+)	(+)	(+)
1/2400	0						
—	—	30'63° C.	0	0	0	0	0
1/300	+	,,	(+)	(+)	(+)	(+)	(+)
1/600	+	,,	(+)	(+)	0	0	0
1/1200	(+)	,,	0	0	0	0	0
1/2400	0						
—	—	30'80° C.	0	0	0	0	0
1/300	+	,,	+	+	+	+	+
1/600	+	,,	+	+	+	+	+
1/1200	(+)	,,	(+)	(+)	(+)	(+)	(+)
1/2400	0						

nativem und 30 Minuten auf 63° und auf 80° C. erwärmten Testes-Virus versetzt. Hiervon wurde auch je eine Kontrollreihe angesetzt. Der Versuch ist in Tabelle 7 wiedergegeben. Es zeigt sich, dass das native Kaninchen-Testes-Virus bis zur Verdünnung von 1/240 selber Hühnerblut agglutiniert, nach Erwärmen auf 63° und 80° C. aber nicht mehr. In dem oberen Drittel der Tabelle zeigt sich überall Agglutination, da sowohl das Karbouwenvirus, als auch das Testes-Virus agglutiniert. Im 2. Drittel der Tabelle finden wir deutlich teilweise Hemmung. Im Testes-Material ist das thermolabile Haemagglutinin zerstört, und die Hemmungsstoffe können sich gegen das Karbouwenvirus auswirken. Diese hemmenden Stoffe sind nicht sehr stark, denn nur die einfache agglutinierende Dosis des Karbouwenvirus wird gehemmt, die doppelte Dosis nur teilweise, doch ist die Hemmungswirkung unverkennbar. Im letzten Drittel der Tabelle sind durch die Temperatur von 80° C. nicht nur die Haemagglutinine sondern auch die hemmenden Stoffe zerstört, und das Karbouwenvirus kann in allen Röhrchen Haemagglutination verursachen.

Hemmungsstoffe finden sich also nicht nur in unwirksamen Viruspraeparaten, sondern auch in wirksamen.

Besprechung der Ergebnisse.

Aus den hier mitgeteilten Befunden ersieht man, dass Pulpas verschiedener Tiere und ebenso Testes-Virus für Haemagglutination und für den Anti-Haemagglutinations-Versuch prinzipiell brauchbar sind. Störend macht sich nur die grosse Labilität bemerkbar. Während Karbouwenpulpa viele Monate lang brauchbar zu sein pflegt und in getrocknetem Zustande selbst noch nach 18 Jahren gute Resultate ergibt, müssen die meisten der hier untersuchten Pulpen so schnell wie möglich nach der Gewinnung benutzt werden. Es zeigte sich auch, dass die 10%igen Suspensionen aus Karbouwenpulpa viel länger unverändert im Eisschrank aufbewahrt werden können, ohne an Wirksamkeit zurückzulaufen, als die verschiedenen anderen Pulpen. Allerdings zeigen sich auch bei der Karbouwenpulpa-Suspension individuelle Schwankungen, denn manche sind nach 3 Wochen noch unverändert, andere aber beginnen schon nach einer Woche schwächer zu werden.

Über Verwendung von Chorionallantois-Material liegen hier keine Erfahrungen vor, auch ist leider die diesbezügliche Literatur im Journal of Hygiene dem Institut nicht zugänglich. Nach den

Ergebnissen im Institut von BURNET scheint dieses Material ausserordentlich gut geeignet zu sein.

Interessant ist fernerhin die Tatsache, dass im Gegensatz zur relativ thermostabilen Karbouwenpulpa die Haemagglutinine in Kaninchentestes deutlich thermolabil sind. Auf diese Abweichung, die sich bei verschiedenen Wirtstieren findet, soll später an anderer Stelle eingegangen werden.

Zusammenfassung.

Pulpa von verschiedenen Tierarten und Testes-Virus eignen sich zur Haemagglutination und zur Feststellung von Anti-Haemagglutinen. Ihre Haltbarkeit ist weniger gut als die der Karbouwenpulpa.

Hemmende Stoffe finden sich nicht nur in unwirksamer Kalblymphe, sondern auch in agglutinierendem Testesmaterial.

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(Aus dem Institut Pasteur, Bandoeng, Java).

HAEMAGGLUTINATION DURCH POCKENVIRUS

8. Die Entwicklung von Antihaemagglutininen durch Vaccinia-Virus verschiedener Herkunft [1])

von

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(Eingegangen am 29 August 1949).

Bei der Herstellung von Vaccine für menschlichen Gebrauch wird in der Regel nur die Virulenz bestimmt, um zu schwache Operationsnummern auszuschalten. Bisher war nicht eindeutig festgestellt, ob die verschiedenen Impfstoffe neben der untereinander oftmals sehr abweichenden Virulenz auch in ihrer immunisierenden Fähigkeit deutliche Abweichungen zeigten. Hierbei kam es an dieser Stelle weniger darauf an festzustellen, wie sich die Hautimmunität entwickelte. Wichtiger schien die Feststellung humoraler Antistoffe zu sein, die sich nach Einverleibung des Vaccinevirus entwickelten.

Am einfachsten ist die Untersuchung der Antihaemagglutinine, und somit wurde untersucht, wie sich diese nach Immunisierung mit der Pulpa verschiedener Karbonwen beim Meerschweinchen (und bei der Ratte) entwickelten. Die Wahl der Antihaemagglutinine als Modell ist einigermassen willkürlich, denn weder sie noch die anderen bekannten humoralen Antistoffe dürften für sich allein den Immunitätsgrad des Individuums bestimmen. Ebenso wie die Revaccination nur eine — obendrein unvollkommene — Vorstellung von der Immunität der Haut geben kann, ebenso ist die Feststellung eines einzelnen der humoralen Antistoffe nichts mehr als der Nachweis, dass die bei der Immunisierung in Wirksamkeit tretenden Zellgruppen des Organismus imstande sind, gegen bestimmte Vaccineantigene Antistoffe zu bilden und diese in die Blutbahn abzugeben. Über die Faktoren der Immunität des

[1]) Mitteilung 7: Antonie van Leeuwenhoek **15**, 167, 1949.

Individuums wissen wir so gut wie nichts. Es ist zwar vorstellbar, dass irgend welche humoralen Antistoffe als Indikator dieser Immunität anzusprechen wären, aber experimentelle Beweise hierfür sind beim Menschen nicht zu erbringen.

Wohl aber liegen Versuche von Fenner und Fenner (3) vor, die in Mäusedörfern die Infektion mit Mäusepocken (Ectromelie) bei gut immunisierten, schlecht immunisierten und bei Kontrollmäusen untersuchten. Nach Immunisierung mit Vaccinevirus auf nasalem Wege wurde zunächst bei allen Tieren der Titer für Antihaemagglutinine festgestellt, worauf zu je 100 männlichen Tieren 15 mit Mäusepockenvirus infizierte Weibchen gesetzt wurden. Tiere mit Antihaemagglutininen erwiessen sich als geschützt. Entwickelten sich nach der nasalen Immunisierung keine Antihaemagglutinine, so verhielten sich die Tiere wie ungeimpfte Kontroll-Mäuse. Ging im Laufe der Zeit der Antihaemagglutiningehalt im Blute zurück, so erwiesen sich diese Tiere auch wieder empfänglich für die Virusinfektion. Fenner und Fenner sprechen sich dahin aus, dass die Antihaemagglutinine, wenn sie nicht etwa sogar den wichtigsten Antistoff für die Beschützung der Tiere gegen Mäusepocken darstellen, auf jeden Fall doch ein ausgezeichneter Index für Anwesenheit oder Abwesenheit der antiinfektiösen Antistoffe sind.

Für die Immunität der weissen Maus gegen Mäusepocken-Virus sind also nach den Untersuchungen von Fenner und Fenner die Antihaemagglutinine ein ausgezeichneter Gradmesser für die Immunität des Individuums. Leider liegen diesbezügliche Untersuchungen oder Beobachtungen naturgemäss für die Variolainfektion des Menschen nicht vor. Auffallend ist allerdings, dass bei der Bestimmung der Antihaemagglutinine bei Variola-Patienten teilweise leichtkranke Personen einen sehr hohen Titer und schwerkranke Personen einen auffallend niedrigen Titer aufweisen. Der günstige bzw. ungünstige Ablauf der Krankheit scheint also wenigstens bei einem Teil der Patienten mit der Titerhöhe in Verbindung zu stehen.

Trotzdem somit beim Menschen noch nicht eindeutig bewiesen ist, inwieweit die Antihaemagglutinine tatsächlich für die Beurteilung des Immunitätszustandes zu verwerten sind, schien uns doch wünschenswert zu untersuchen, ob verschiedene Vaccinelymphen mit deutlich abweichender Virulenz auch Abweichungen in ihrer Fähigkeit Antihaemagglutinine zu bilden zeigten. Im

Zusammenhang hiermit war es weiterhin interessant festzustellen, wie sich Vaccine anderer Provenienz (z.B. von Kuh, Kaninchen, Meerschweinchen usw.) in dieser Hinsicht verhielt.

Methodik.

Vaccine: Die hier für die menschliche Impfung gebräuchliche Karbouwenvaccine wurde 3 Tage nach Impfung geerntet und ohne weitere Verarbeitung bis zum Gebrauch bei —15° C. aufbewahrt. Die Pulpa vom Kalb und von der Kuh (Indiasche Rasse) war gleichfalls 3 Tage nach Impfung gewonnen. Vaccine vom Schaf, Kaninchen, Meerschweinchen und Affen (*Macaca irus mordax*) wurde 4-5 Tage nach der Impfung gebraucht, da hier die Entwicklung etwas langsamer vor sich ging. Testesvaccine wurde 5 Tage nach Infektion gewonnen.

Virulenzbestimmung: Die Virulenz wurde nach der Methode von Gins bestimmt durch Einreiben verschiedener Verdünnungen des Materials in skarifizierte Augen von Meerschweinchen. Diese Methode ergibt Minimalwerte, ist aber in Vergleichsreihen ausserordentlich zuverlässig.

Immunisierung: Das Impfmaterial wurde im gewöhnlichen Mörser möglichst fein gerieben und mit physiologischer Kochsalzlösung zu einer 10%igen Suspension verarbeitet. Diese wurde sodann 10 Minuten lang bei 2000 Touren zentrifugiert und abgegossen. Die Meerschweinchen erhielten hiervon 0,5 ccm intraperitoneal injiziert, Ratten (bunte Rasse) die gleiche Menge. Die Meerschweinchen wurden nach 12 Tagen bzw. 20 Tagen und die Ratten nach 13 Tagen untersucht.

Titerbestimmung des Serums: Das inaktivierte Serum wurde auf seine Fähigkeit untersucht, die Agglutination von Hühnerblut durch die doppelte wirksame Dosis Karbouwenpulpa zu hemmen. Die Technik findet sich bei Collier (1). Es sei nur kurz erwähnt, dass wir die letzte Zeit ausschliesslich 1% Pferdeserum-Kochsalzlösung mit Formalinzusatz als Verdünnungsmaterial benutzen. Die Versuche wurden während der Regenzeit ausgeführt.

Immunisierung mit Karbouwenpulpa.

Verschiedene Operationsnummern von Karbouwenpulpa von meistenteils mittlerer Virulenz wurden bei je einer Serie Meer-

schweinchen in der Menge von 0,5 ccm einer 10%igen zentrifugierten Suspension intraperitoneal injiziert. Eine Pulpa war sehr schwach virulent (1/1000), eine weitere hoch-virulent (1/200.000). Nach 12 bzw. 20 Tagen wurden die Meerschweinchen untersucht. Von den bei jeder Serie gefundenen Antihaemagglutininen wurden jeweils die Mittelwerte berechnet. Die Virulenzeinstellung erfolgte am gleichen Tage wie die Immunisierung, auch wurde am gleichen Tage der Titer des Virus auf Haemagglutination untersucht. Die Befunde sind in Tabelle 1 zusammengestellt.

Tabelle 1.

Immunisierung von Meerschweinchen mit verschieden virulenter Karbouwenpulpa: Ausbildung der Antihaemagglutinine. (Immunisierende Dosis = 0,5 cc 10% intraperitoneal).

Karbouwenpulpa:	Virulenz für Cavia Augen:	Titer der Haemagglutination:	Antihaemagglutinine nach 12 Tagen		Antihaemagglutinine nach 20 Tagen	
			Zahl der Caviae:	Durchschnitt: (reziproker Wert)	Zahl der Caviae:	Durchschnitt: (reziproker Wert)
1	1/1000	1/2560	—	—	10	960
2	1/10.000	1/2560	10	432	5	1408
3	1/10.000	1/1280	8	780	7	1920
4	1/10.000	1/1280	5	912	2	1280
5	1/10.000	1/1280	6	387	3	480
6	1/20.000	—	6	683	4	720
7	1/20.000	1/640	10	352	3	507
8	1/20.000	1/640	12	418	8	2000
9	1/50.000	1/2560	7	229	4	720
10	1/50.000	—	7	628	4	1512
11	1/50.000	1/2560	9	431	5	1280
12	1/50.000	1/2560	5	1032	3	1173
13	1/50.000	1/1280	8	512	4	560
14	1/50.000	1/1280	13	569	7	457
15	1/50.000	1/2560	7	571	6	527
16	1/200.000	1/640	—	—	12	4533

Während die Virulenz der verschiedenen Pulpen von 1/1000 bis 1/200.000 schwanken, ist die Haemagglutination bedeutend regelmässiger, sie schwankt nur von 1/640 bis 1/2560. Aber auch die Durchschnitts-Titer der Antihaemagglutinine innerhalb der ver-

Tabelle 2.

Immunisierung von Meerschweinchen mit verschiedenen Arten von Pulpa: Ausbildung der Antihaemagglutinine. (Immunisierende Dosis = 0,5 cc 10% intraperitoneal).

Pulpa von:	Virulenz für Cavia-Augen:	Titer der Haemagglutination:	Versuchsserie No.:	Antihaemagglutinine nach 12 Tagen		Antihaemagglutinine nach 20 Tagen	
				Zahl der Caviae:	Durchschnitt: (reziproker Wert)	Zahl der Caviae:	Durchschnitt: (reziproker Wert)
Kalb A	1/1000	1/40	1	16	363	15	707
			2	8	70	7	283
			3	12	32	6	216
Kalb B	>1/1000	1/320	1	15	45	13	16
			2	8	30	8	147
			3	12	66	6	184
Kalb C	1/100.000	1/160 (+)	1	17	566	9	1707
			2	8	540	7	2418
Kalb D	1/50.000	1/80 (+)	1	19	835	11	1767
			2	7	297	5	1344
Kuh 381	1/5000	1/640 (+)		8	440	2	1360
Kuh 382	1/100.000	1/320 (+)		6	533	3	666
Kuh 383	1/100.000	1/160 (+)		2	800	—	—
Kuh 384	1/200.000	1/320 (+)		1	160	—	—
Schaf I	>1/1000	>1/20		15	31	9	7965
Schaf II	1/5000	>1/20		15	69	8	840
Kaninchen	1/50.000	1/640		15	2475	12	2093
Cavia	—	1/1280		17	2071	13	6218
Affe I	1/500.000	1/1280		2	2560	2	2400
Affe II	1/200.000	1/1280		1	5120	—	—
Affe in Glycerin	1/50.000	1/80		5	580	5	992
Kaninchen-Testes							
a	—	>1/20		19	55	15	87
b	—	1/960		16	1088	11	6517
c	1/20.000	1/320		16	62	9	2595
d	—	1/320		10	216	4	200
e	—	1/160		8	400	4	800

(+) Vgl. Text.

schiedenen Serien schwankt nicht allzusehr. Bei der Untersuchung nach 12 Tagen liegen die Werte zwischen 1/229 und 1/1032. Hierbei muss noch darauf hingewiesen werden, dass diese beiden Extremwerte zufällig durch zwei Pulpen erreicht werden, die genau die gleiche Virulenz aufweisen. Bei der Untersuchung nach 20 Tagen liegen die Antihaemagglutinine zwischen 1/480 und 1/4533. Es ist wohl nur ein Zufall, wenn dieser Maximalwert durch das am stärksten virulente Material erzielt wurde, denn der zweithöchste Durchschnittswert wird bei einer Pulpa mit der Virulenz von 1/10.000 gefunden.

Im Grossen und Ganzen kann man also sagen, dass kein deutlicher Zusammenhang zwischen Virulenz und immunisierender Fähigkeit (wenigstens was die Erzeugung von Antihaemagglutininen angeht) zu sehen ist. Alle 16 untersuchten Operationsnummern waren imstande, in ungefähr der gleichen Weise Antistoffe zu erzeugen.

Immunisierung mit Vaccine von verschiedenen Tieren.

Nicht so gleichmässig wie die bisher mitgeteilten Versuche verliefen die Immunisierungen mit verschiedenen Pulpen von anderen Tierarten, die in der Tabelle 2 zusammengestellt sind.

Bei Kalb A und B handelte es sich um altes Material von sehr niedriger Virulenz, wodurch nur in der ersten Versuchsserie relativ hohe Titer erreicht wurden. Erwähnt sei noch, dass bei Kalb B der Wert von 1/320 bei der Haemagglutination für Kälbermaterial sehr hoch erscheint, dass hier aber wohl kaum eine spezifische Haemagglutination anzunehmen ist. Antisera von Meerschweinchen und Ratte hatte auf diese Haemagglutination keinen Einfluss, sodass man eine unspezifische Reaktion annehmen muss.

Die Pulpa von Kalb C und D war vollkommen frisch und immunisierte bei hoher Virulenz. sehr gut. Die Haemagglutinationswerte lagen sehr hoch, waren aber schon nach wenigen Tagen völlig verschwunden.

Die Pulpa von den Kühen war sehr virulent. immunisierte aber etwas weniger gut, als die Pulpa der Kälber. Auch hier waren die Haemagglutinationswerte bereits nach wenigen Tagen gänzlich verschwunden.

Die Pulpa der beiden Schafe war in beiden Fällen nur wenig virulent, trotzdem war die bei Meerschweinchen durch Pulpa von

Schaf I erzeugte Immunität nach 20 Tagen ausserordentlich gut, obwohl sie nach 12 Tagen noch sehr niedrig gewesen war. Die Pulpa beider Schafe agglutinierte nicht.

Die Pulpa von Kaninchen und Meerschweinchen immunisierte sehr gut, schon nach 12 Tagen waren sehr hohe Werte erreicht.

Mit Affenpulpa wurden nur wenige Meerschweinchen immunisiert. Die frische Pulpa ergab recht hohe Immunisierungswerte, glycerinisierte Pulpa (ca. 6 Wochen alt) weniger gute. Die Virulenz der Affenpulpa lag sehr hoch.

Die Immunisierungserfolge mit Vaccine von Kaninchen-Testes waren verschieden gut. Testes *a* ergab keine Haemagglutination und immunisierte sehr schlecht, Testes *b* dagegen agglutinierte gut und erzeugte sehr hohe Immunität. Testes *c*, *d* und *e* lagen dazwischen.

Zusammenfassend sieht man aus der Tabelle, dass die verschiedensten Pulpen und Kaninchen-Testes alle imstande sind, wenn auch in verschieden starken Masse, Meerschweinchen zu immunisieren. Hierbei erreichen in manchen Fällen die Antihaemagglutinine sehr hohe Titer, die in den hier mitgeteilten Versuchen bis zu 1/7965 gingen.

Noch besser als Meerschweinchen eignen sich Ratten zur Er-

Tabelle 3.

Immunisierung von bunten Ratten mit verschiedenen Arten von Pulpa: Ausbildung der Antihaemagglutinine.
(Immunisierende Dosis = 0,5 cc 10% intraperitoneal).

Pulpa von:	Virulenz für Cavia-Augen:	Titer der Haemagglutination	Antihaemagglutinine nach 13 Tagen	
			Zahl der Ratten:	Durchschnitt: (reziproker Wert)
Schaf I	>1/1000	>1/20	9	1386
Schaf II	1/5000	>1/20	9	5120
Kaninchen	1/50.000	1/640	9	7395
Affe I	1/500.000	1/1280	3	8190
Affe II	1/200.000	1/1280	3	5923
Affe in Glycerin	1/50.000	1/80	6	3093
Kaninchen-Testes	1/20.000	1/320	10	3968
Karbouw	1/50.000	1/1280	19	3568

zeugung von Antihaemagglutininen. Infolge Tiermangels konnten aber nur wenige Serien immunisiert werden. Die Durchschnittswerte sind in der Tabelle 3 zusammengestellt. Die niedrigsten Werte fanden sich nach der Immunisierung mit Pulpa von Schaf I, das auch die niedrigste Virulenz aufwies. Die höchsten Werte ergab Immunisierung mit Affen-Pulpa, die zugleich auch die höchste Virulenz aufwies. Prinzipiell liegen alle Werte in ungefähr der gleichen Grössenordnung.

Besprechung der Ergebnisse.

Für die Praxis der Vaccine-Herstellung ist vor allem die Tatsache von Interesse, dass unabhängig von der Virulenz die immunisierende Fähigkeit der verschiedenen Operationsnummern von Karbouwen-Pulpa einigermassen in der gleichen Grössenordung liegt. Dies ist insofern von Bedeutung, als gelegentlich seitens der Praxis die Vermutung geäussert wird, dass vielleicht eine oder die andere Nummer trotz guter Impferfolge auf dem Arm weniger Immunität erzeugen könnte als die andere. Diese Vermutung wird vor allem in Zeiten schwerer Epidemien geäussert, wo gelegentlich auch bei „erfolgreich" revaccinierten Personen Pocken zum Ausbruch kommen. Die hier geschilderten Versuche lassen aber erkennen, dass Pockenvaccine vom gleichen Spender und unter gleichen Bedingungen gewonnen auch ungefähr den gleichen immunisierenden Wert hat, wenigstens was die Erzeugung von Antihaemagglutininen betrifft.

Rein theoretisch war dies ja einigermassen zu erwarten, handelt es sich bei der Vaccination ja um eine Infektionsimpfung, die im Organismus zum Haften kommen muss. Kommt es bei genügender Virulenz überhaupt dazu, so kommt es bei dem nunmehr einsetzenden Immunisierungsvorgang nicht mehr auf die ursprüngliche Virulenz des Virus an, sondern nur noch auf die Eigenschaften des im Körper neugebildeten Virus und auf die Reaktionsfähigkeit der beim Immunisierungsprozess beteiligten Zellsysteme. Man darf wohl den Schluss ziehen, dass zur Vaccination jedes beliebige Material zu gebrauchen ist, wenn es nur genügend virulent ist um sich im Körper ausreichend zu vermehren. Die Faktoren, die diesen Vorgang befördern, wie beispielsweise Hyaluronidase bildende Bakterien usw, ändern nichts an dieser prinzipiellen Auffassung.

Zusammenfassung.

16 verschiedene Pulpen vom Karbouw von sehr auseinanderlaufender Virulenz erzeugten beim Meerschweinchen Antihaemagglutinine von ungefähr gleicher Grössenordnung.

Pulpa vom Kalb, Kuh, Schaf, Kaninchen, Meerschweinchen und Affen und Kaninchen-Testes-Virus erzeugten beim Meerschweinchen gleichfalls deutliche Antihaemagglutinine. Analog verliefen Versuche bei der Immunisierung von Ratten.

Zur Vaccination gegen Pocken dürfte jedes beliebige Material brauchbar sein, wenn es nur genügend virulent ist und zum Haften kommt.

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THE CONCEPT „ASSOCIATION" IN MYCOLOGY

by

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Mycological and technological experience in the Centraalbureau voor Schimmelcultures has shown that the flora of spoilage fungi occurring on different substrates of organic origin in decay will be composed of different species. In a given substrate the species of micro-organisms, active in decay, are fairly constant, although under the influence of changes in temperature and humidity, alternations may occur.

If, by application of a proper cultivation technique the total microflora originating from the air and surroundings and active in spoilage of a given material is analysed, the composition of the microflora will be noted to be very constant. This specific microflora belonging to a special substrate, I propose to term a s s o c i a t i o n. The term "association" is a quite familiar one in plant sociology, where it has been noticed that soils, differing in chemical and physical properties, will give rise to different vegetations. More recently we met the concept association also in description of toadstool-combinations, found in certain territories.

We may compare biogenic substrates with soils, since the former too are selective media, whereon — dependent on chemical and physical properties — some organisms may thrive, whilst others do not get a chance to develop.

Investigations along this line have been carried out in our laboratory for years. As a matter of fact owing to our specialization, we have always focussed our attention on the fungal elements of the association, although characteristic bacteria have been regularly noticed too.

Some interesting examples from our experience may be given.

In decaying w o o d, occurring in soils, the lignase- and cytase-containing fungus *Trichoderma viride* invariably dominates the situation in the first stage; later on, when saccharification is proceeding, Penicillia join the *Trichoderma* species.

On s t u b b l e s, occurring in cereal fields after the harvest, black fungi (*Dematiaceae*) dominate, among which *Cladosporium herbarum* is the most constant one. This fungus is generally accompanied by *Alternaria* and *Stemphylium* species.

On j u t e, s i s a l, c o c o s and allied fibres, an association of *Mucor racemosus*, *Mucor spinosus* and *Penicillium* species is found. The fungi, attacking c o t t o n and l i n e n, thus giving rise to „the weather" in the tissues, however, are: *Cladosporium herbarum*, *Alternaria tenuis*, *Pullularia pullulans* and some *Stemphylium* and *Stysanus* species. An allied association is found on p a p e r: *Alternaria chartarum*, *Chaetomium* species, *Stysanus stemonitis* and *Cladosporium herbarum*.

On w o o d p u l p a very special association, comprising *Phialophora* species and yeast-like fungi of the genus *Hyalodendron*, occur.

Rough t o b a c c o tends to a type of spoilage, wherein *Monilia* species dominate (*Monilia cerebriforme*, *Monilia macrospora*, *Monilia diversispora*, *Monilia mediacensis*, *Monilia tomentosa* and *Monilia microspora*).

L e a t h e r, as is well known, is under moist conditions, attacked by fungi. In these cases invariably *Penicillium solitum* and *Penicillium corylophilum* are present. On softer types of leather (such as are used in glove manufacture) *Mucor* species join the Penicillia.

Moist c o c o n u t-mesocarp may be attacked by *Aspergillus niger* and *Rhizopus* species; in c o p r a-spoilage moreover *Aspergillus glaucus* and *Aspergillus cinnamomius* form part of the association. From spoiled m a r g a r i n e and b u t t e r *Margarinomyces atrovirens*, *Margarinomyces Bubaki*, *Margarinomyces luteoviridus*, *Cladosporium butyri* and *Tilachlidium butyri* were isolated; they are the cause of black spots.

Musty Dutch r y e-f l o u r s mostly appeared to have been attacked by *Rhizopus* and *Aspergillus* species. Mouldy p a p r i k a from balkanian countries, invariably showed an association of *Rhizopus arrhizus*, *Rhizopus nigricans*, *Aspergillus flavus* and *Aspergillus niger*. The Aspergilli developed at temperatures from

25° to 30° C., whereas the *Rhizopus* species appear between 15° and 20° C.

A very striking example of an association involved by selection is that on mouldy oranges. We always found the two species *Penicillium italicum* and *Penicillium olivaceum*, independent of the type of the fruit and the country, where they were grown.

On rotten apples in cellars we often found one single fungus only, *i.e. Penicillium expansum*. This same fungus is nearly always to be found on decaying bulb scales of tulips.

Summary.

Referring to the use of the same concept in plant sociology, the term association is proposed for the specific combination of micro-organisms, found in decaying biological substrates. A number of representative associations is given. It seems desirable to introduce „association", which is familiar to botanists, to the microbiologists.

(From the Central Institute for Nutrition Research T.N.O., Utrecht, Holland and Centraal Bureau voor Schimmelcultures, Baarn, Holland).

THE PHYSIOLOGY OF MICROBIAL SPOILAGE IN FOODS

by

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Introduction.

The practice of microbial spoilage of foods and allied substrata has revealed that only a very limited part of the microorganisms, present on the substrates, participates in the deterioration process and thus gives rise to a very s p e c i f i c spoilage type for any variety of foods (28, 67, 51, 72).

A few examples may be mentioned. Practically independent of the infecting microorganisms, a commodity rich in protein, will be subjected to putrefactive processes if it spoils under normal conditions. On the contrary: most vegetable foods rich in insoluble carbohydrates are decomposed by moulds when stored under inadequate conditions. Finally, substrates, containing much sugar, such as fruit juices and sirups tend to fermentation.

Even within the classes mentioned before, a high specificity exists. For example, oranges from geographically fully different sources always show an analogous deterioration, wherein certain species of green Penicillia dominate (72).

The second author has proposed to name this highly specific selection of the microflora which governs the spoilage picture, the a s s o c i a t i o n of a food (72). This association is determined by intrinsic, extrinsic and implicit factors. The way in which these parameters influence the origination of the association, will be analysed in this paper.

It must be stressed, that these considerations are not limited to the associations met with in decaying foods, but have a more general character and mutatis mutandis may be applied to the origination of the specific florae found e.g. in the various parts of the digestive tract of animals.

Intrinsic parameters of spoilage.

Chemical composition of the substrate.

The chemical composition of a spoiling substrate is decisive for the numerical importance of nutrients, necessary for the development of various microorganisms. Thus it may be understood, that e.g. in the spoilage of potato starch a very limited part of the ubiquitous spoilage microorganisms will play a role: in this substrate only minute traces of nitrogen and salts are present and therefore only a group of pronounced nitrogen-autotrophic microorganisms will be able to develop.

Another very interesting example of the decisive influence exerted by the nutrient-composition of a substrate is an investigation by Challinor et al (12). These workers established that fruit juices, which had been subjected to an ion-exchange treatment and thus were deprived of a part of the nutrients originally present, only permitted very poor growth to yeasts.

A second important set of factors governed by the composition of a substrate, are its pH and buffering power. The selective influence, exerted by the pH, is illustrated by the fifth section of figure 1. It is clear, that substrates with relatively low pH-values (e.g. fruits) will not be invaded by such weakly acid-tolerant microorganisms as the putrefactive bacteria and inversely: that meat with its pH of about 7 normally will not be attacked by yeasts or lactobacteria.

For the further course of the deterioration the buffering power plays an important role. If the latter is high, the spoilage microflora will be fairly constant, while a low buffering power may be the cause of a spoilage picture that changes essentially during the decomposition process.

Finally the composition of a substrate accounts for the absence or presence of antimicrobial substances. Some essential oils (31, 14, 41, 71, 42, 60, 20) and glycosides (69, 11, 35, 33) occurring in plant substrates, as well as the protein factor of animal origin lysozyme (65, 1) have been shown to exert a more or less specific bacterio- or fungistatic action. If foods, containing these substances, spoil, they will mostly be attacked by special, resistant strains.

The importance of some naturally occurring lipids as regulators of microbial spoilage is not yet perfectly clear (53, 64, 57, 58).

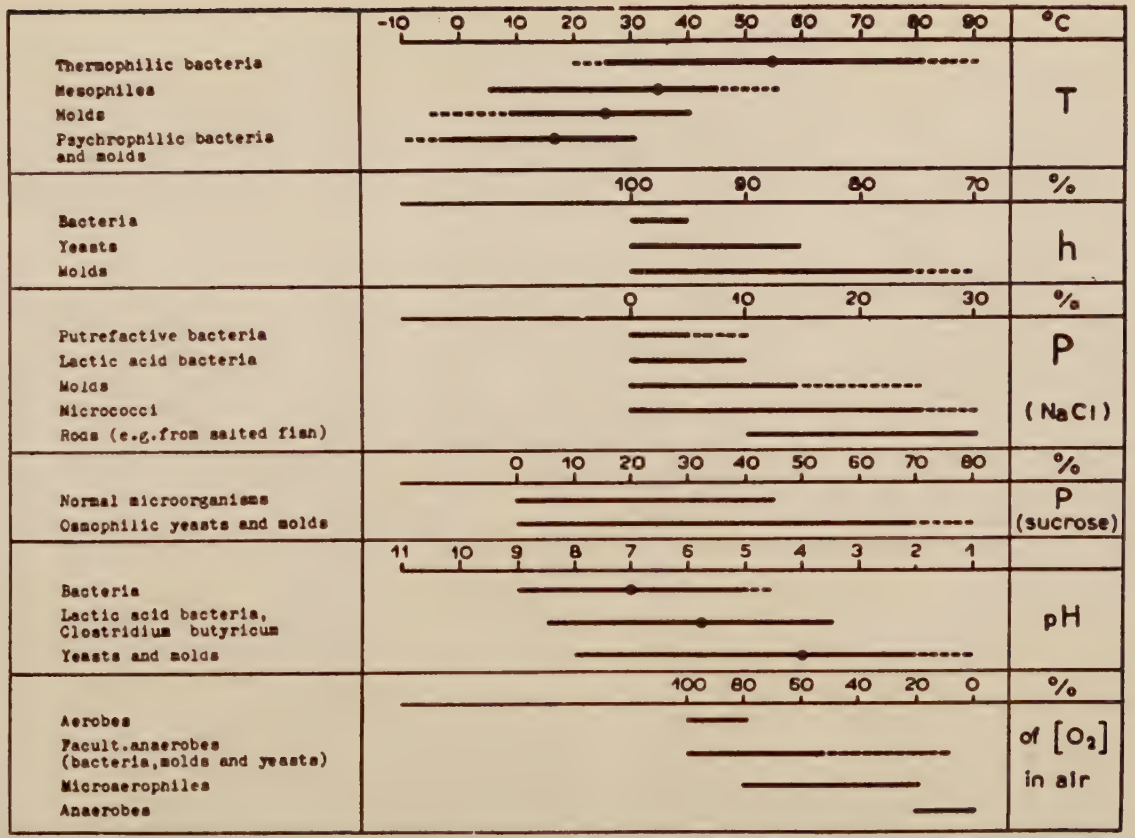

Fig. 1. Parameters governing the development of the most important microorganisms, active in spoilage of biological substrata (Data taken from references nr 5, 7, 8, 9, 23, 26, 27, 30, 34, 37, 39, 45, 63 and 66).

One might be inclined to group the water content of a substrate among the compositional intrinsic factors of spoilage. Since the water content is no independent characteristic of a substrate but is strongly influenced by the relative humidity, existing during storage (47), it is preferred to consider the water activity in a food as an extrinsic factor.

Processing of the substrate.

It needs no further comments that the radical treatments of vegetables, which yield products of the sauerkraut- or pickle-type (16, 10, 52), essentially change the natural microflora of the raw materials. But even the more common processing types as heating or extraction, exert a considerable influence.

One of the most extreme examples is the smoke-curing of meat-products. Here JENSEN (36) found, that for the dominant part the action of smoking is bacteriostatic, since treated commodities still tend to superficial mold-attack, but do not show any appreciable bacterial spoilage.

A similar effect was noticed by the present authors in the course of an investigation on spoilage of paprika (sweet red peppers = *Capsicum annuum* L. (6, 4)). Brands originating from balkanian countries and Spain resp., did not show a significant difference in gross chemical composition, but nevertheless as a rule were attacked by different types of microorganisms. It may be assumed, that this different behaviour is caused by the fairly divergent technique of drying of the pods in the areas mentioned.

Extrinsic parameters of spoilage.

Infection.

In order to become infected with the microorganisms belonging to its association, it is imperative that a substrate has sufficient contact with the natural depots of ubiquitous microorganisms: dust, soil, water, other substrates, etc. Although this extrinsic parameter therefore generally is of paramount importance, it nevertheless needs no discussion here, since industrial practice reveals, that in spontaneous microbial spoilage-processes, infection of a food never is a limiting factor.

Water vapour pressure during storage.

Whether a given microorganism can attack (i.e. multiply in) a certain substrate under a given set of conditions depends on the

osmotic pressure prevailing in the latter (70, 25); cf. sections 3 and 4 of figure 1.

For practical purposes the expression of spoilage chances in terms of osmotic pressure is not very convenient. Now thermodynamics state, that the osmotic pressure (P) of a system is related to the more accessible parameter: water vapour pressure (π) by the equation:

$$P = a\,d\,T \ln \frac{\pi_0}{\pi} \quad \ldots\ldots\ldots\ldots \quad (1)$$

where T = absolute temperature

π_0 = vapour pressure of pure water at $T°K$

d = density of the system

a = constant.

Relation (1) is mostly written as:

$$P = a\,d\,T \ln \frac{1}{h} \quad \ldots\ldots\ldots\ldots \quad (2)$$

where h = relative water vapour pressure or relative humidity of the system, since the latter is very little dependent on temperature.

The second section of figure 1 illustrates at which h-values development of the various common spoilage organisms starts. It is evident that the humidity of the atmosphere exerts a pronounced selective influence on the association which presents itself on a decaying food. At lower humidities no development of yeasts and bacteria is possible and any association occurring in this range will be purely fungal (62, 26). But among the fungi a further selection is possible. Although $h = 0.75$ generally is the lower limit of mold development (66, 21, 27, 43) there exists a group of so-called xerophiles (e.g. *Aspergillus glaucus*) whose germination is only checked by relative humidities below 0.70 (61, 63, 22) and which consequently will dominate under the relatively driest conditions.

At higher humidities yeasts and bacteria get their chance. Owing to the circumstance, that they generally develop at a higher rate than the fungi, they will gradually dominate the spoilage picture when h is increasing over 0.90.

Food manufacturers generally are most interested in what is called the "alarm water content" of commodities, i.e. the lowest water content

which permits microbial spoilage. In order to recalculate humidities to water contents, we are in want of the relation between water content and humidity:

$$W = f(h, T) \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

This function, the water sorption isotherm, strongly varies for different substrates, since it depends on the way, in which the water is bound by the dry substance (47). In cellulose e.g. the water binding is poor and thus a greater part of the water present will be "free" (24) and exert a relatively high vapour pressure. On the contrary in starch the water is strongly bound and therefore shows a lower vapour pressure. Figure 2 which gives the water sorption isotherms for a set of important foods at 20° C., fully illustrates this.

With the help of figures 1 and 2 we can treat spoilage problems in terms of alarm water contents.

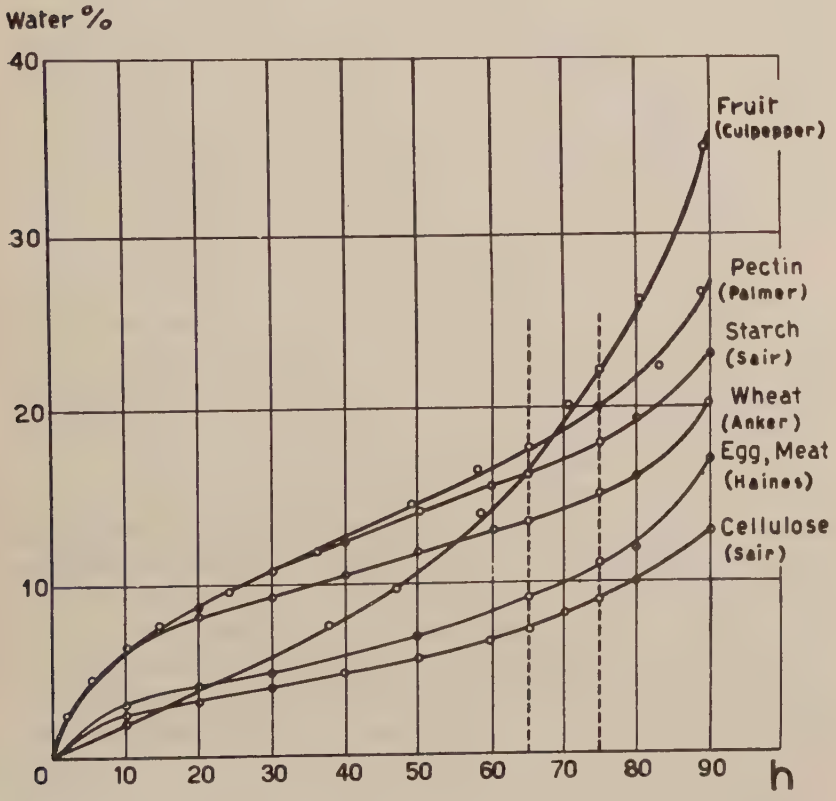

Fig. 2. Water sorption isotherm at 20° C. of some outstanding foods (Data taken from references nr 2, 15, 26, 50 and 59).

Section 2 of fig. 1 shows that generally at h $\sim$ 0.65 no spoilage will occur. Figure 2 reveals that for wheat flour e.g. h = 0.65 corresponds to a water content of 13.5 %. Hence below 13.5 % of moisture no spoilage can occur in flour, i.e. the alarm water content of flour is 13.5 %.

Temperature of storage.

The temperature ranges, wherein development of the most important classes of spoilage microorganisms occurs, are given by section 1 of figure 1.

Since the range —10 to +70° C. is covered by the various types of ubiquitous germs, under common conditions a food will inevitably spoil provided the humidity is feasible. The character of the dominant microorganisms will, however, essentially vary with varying temperature. At "room temperatures" (15 to 25° C.) molds, yeasts and mesophilic bacteria, e.g. *Pseudomonas* and *Streptococcus* species will govern the spoilage picture, while under tropical conditions the common bacteria as the Bacilli and coliforms will dominate. At cold storage temperatures some special strains: the psychrophiles e.g. *Achromobacter* and *Pseudomonas* species (39, 3) and the mold *Cladosporium herbarum* (8) come to the front.

Oxygen partial pressure.

Section 6 of figure 1 illustrates the selective effect of oxygen partial pressure. A very illustrative example of this effect is that the microorganisms, which sometimes show activity in canned foods, form a special class of thermo-resistant gram-positive rods (5) which under any other set of conditions do not get an opportunity to govern the spoilage picture.

It may be stressed in this connection that the older concepts of the obligate aerobiosis of fungi are to be cancelled in the light of the results more recently obtained by GOLDING and collaborators (23, 45). These workers succeeded in confirming and enlarging the observations of BROWN (9) who as early as 1922 showed that fungi, active in fruit rot processes, developed very well at oxygen partial pressures of the order of 50 mm Hg. Hence, although mold spores prefer to develop on aerobic surfaces if they are free to do so, flourishing of fungi is possible even under pronounced anaerobic conditions.

Exposure to sunlight.

It is evident that in field spoilage of agricultural products domination of special strains can be expected, since the irradiation by ultraviolet light will kill or at least inactivate a great number of common species.

Implicit parameters in microbial spoilage.

In the preceding sections we demonstrated how intrinsic and extrinsic factors effectuate a selection in the development of the microorganisms present on a substrate, thus only permitting a limited choice of organisms to participate in the spoilage process. This selected choice nevertheless may not develop as a totality, viz. in cases when some of its components get an opportunity to dominate and suppress others due to difference in rate of development or phenomena of antagonism and synergism.

The influence exerted by the latter factors is strongly determined by the microorganisms which belong to the primary selection. Thus the effect of these factors is not an independent one and therefore we suggest to nominate them — in analogy to the corresponding concept of mathematics — implicit parameters of microbial spoilage.

Rate of development.

The "rate of development" of a strain is only an implicit parameter in so far as it is defined as occurring under optimal conditions. Any retardation of growth due to sub-optimal environment has been included under the heading extrinsic parameters.

It is a well established fact, that some microorganisms show a rapid (optimal) growth, while others are tardy. These differences are partly caused by essential biochemical characteristics of the various bacteria, but moreover depend on accidental factors, e.g. the origin of a given strain (29). We shall not treat this subject more in detail and limit ourselves to establishing that among the normal saprophytes significant differences in rate of development may be noticed. Rods as *B. subtilis* or *P. vulgaris* e.g. develop with great rapidity, while fungi and lactobacteria generally are slower.

Synergism.

Synergism is defined as the phenomenon wherein a certain microorganism or group of microorganisms:

1) enables others to develop;
2) stimulates the development which already prevails.

An example of the first type of synergism is the development of microorganisms (e.g. of *Lactobacillus* species), enabled by the synthesis of factors of the vitamine B complex or other oligodynamic nutrients (40, 55) by other strains of microorganisms (e.g. yeasts).

The second type is more common. It is often noticed that a microorganism effects a change in the pH of the medium (either a decrease due to the formation of acids or an increase, caused by decarboxylation reactions) which is favourable to other components of an association. Further it occurs in many instances, that fungi make a medium more suitable for the development of certain other fungi or bacteria by hydrolyzing a part of the cellulose or other cell wall constituents to sugars (48).

Antagonism.

A group of microorganisms may check the development of other components of the association to which they belong in one of the following ways:

1) rendering the medium less favourable by changes in the pH or preferential consumption of nutrients;
2) formation of antibiotic substances.

The latter may be of different types. Simple molecules as alcohols (13, 54, 75) and fatty acids (37, 38, 32, 74, 46) which are formed by numerous saprophytic microorganisms exert a strongly inhibitory action on many other common spoilage organisms.

The more complicated structures as the penicillins and antibiotics from Actinomycetes have been extensively reviewed (68) and hence need no further treatment here.

Finally some interesting protein-antagonists have been studied more recently (56, 73, 49, 44, 19). Streptococci of the lactis-cremoris type may produce substances antibiotic for strains of the same group, which are commonly used as "starters" in dairying and thus may cause highly unwanted "slowness" in the production of lactic acid.

Discussion.

It appears that the association which dominates the spoilage of a given substrate under specified external conditions, has a very complex genesis. If therefore aspects of spoilage processes are to be studied, this has to be carried out in the substrate under investigation. Only in relatively few, isolated cases this has actually been done (17, 18, 31, 37, 46). Mostly researches on food spoilage are carried out with what are called "representative strains" in agar media. Proceeding in this way the essential error is made, that the influence of the intrinsic factors, associated with the food itself and of the implicit parameters is fully neglected. Since the latter constitute $^2/_3$ of the variables, active in actual spoilage, serious deformations of the real situation may be effected by such experiments.

In some cases the food itself was actually chosen as a medium, but by "accelerating" the spoilage processes by elevated temperatures or humidities again an essential deformation was introduced. A slight acceleration of spoilage processes can be tolerated for experimental work, but such must be limited to the extent deduced from a close consideration of the deterioration as it occurs in practice.

Summary.

1. In microbial spoilage of foods only a very limited selection of the actual infection (the association of the substrate) plays a role.

The genesis of the association is influenced by intrinsic, extrinsic and implicit factors.

2. Intrinsic parameters include chemical composition and processing of the substrate. The most important extrinsic parameters are activity of water, temperature, oxygen pressure and irradiation prevailing in the system during spoilage.

3. Among the microorganisms, whose development is made possible by the other parameters, further selection is effected by what were nominated implicit factors, i.e. differences in rate of development and synergism, resp. antagonism.

4. The complexity of the genesis of the association makes it imperative that researches on microbial spoilage are carried out in the substrate under investigation and under external conditions which do not differ essentially from the situation in practice.

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A NEW APPARATUS FOR GRINDING BACTERIA

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The apparatus described in this paper brings an improvement of that devised by KALNITSKY, UTTER and WERKMAN (1) for grinding bacterial cells.

In order to obtain cell-free enzyme preparations, several instruments have been made for the mechanical disintegration of bacteria. MUDD, SHAW, CZARNETSKY and FLOSDORF (2) constructed the freezing ball-mill, in which the bacteria made brittle by freezing were crushed by steel balls. BOOTH and GREEN (3) constructed a wet crushing mill, in which the bacteria were crushed by steel rollers in a steel cylinder.

In the apparatus of KALNITSKY et al. (1) the bacterial cells mixed with glass powder are ground by being gently forced between two closely fitting, concentric, ground glass cones, the inner one of which is rotated.

However this apparatus has several disadvantages of which we mention:

1. The large quantities of bacterial cells required for each operation; no less than 10—15 grams of wet bacterial cells are considered to be necessary.

2. The use of glass powder which has to be prepared beforehand. The milling and sieving of this powder takes a considerable time. If steel balls are used the powder has to be purified by treatment with hydrochloric acid and subsequent washing. Glass powder, especially in a dry state, is a dangerous substance to work with; the wearing of a dustmask is advised (1, 4).

3. The large amount of glass powder used (a ratio of 1 : 2 of

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bacterial cells to glass powder is recommended) has a considerable adsorbing capacity. We calculated that the total surface of the glass powder, with particles of a diameter of 2 micron, necessary for one milliliter enzyme suspension amounts to 2—3 m^2 of glass surface. As a matter of fact KALNITSKY et al. state (1) that, if a larger ratio of glass powder to bacterial cells is used, the obtained enzyme solutions are less active.

4. The extruded material is exposed to the air. Consequently grinding under sterile conditions is impossible and also the grinding in an inert gas, e.g. nitrogen. Yet in our experiments with luminous bacteria the latter has proved to be of great importance.

5. The instrument does not work automatically, but the glass-bacteria paste has to be pushed in by a plunger operated by hand.

In the instrument described below all these disadvantages have been overcome.

The apparatus is fully automatical; while working it can be left alone. No glass powder is used and the grinding is carried out under sterile conditions and if necessary in a nitrogen atmosphere. The essential feature of the instrument (by which all these above mentioned difficulties could be avoided) is the reduced grinding surface. All bacteria have to pass a single circular line, where they are crushed in one operation. This is achieved by replacing the inner rotating cone of KALNITSKY's apparatus by a rotating ball-shaped surface and by widening the outer cone's angle. It is evident that the contact region of the outer cone and the inner ball surface consists of a very narrow circular zone, almost a line. The consequence is, that the pressure exerted on this line of contact between the two glass parts is very high with a low total pressure on the outer cone. The film of cell suspension between the two glass parts is very thin and in fact thinner than the dimension of a cell.

Thus it is hardly possible for a cell to pass the line of contact without being crushed, which makes the use of glass powder superfluous. The cells are at the same time emptied which can be clearly seen, when a yeast suspension which has passed the apparatus is examined under the microscope.

The time necessary for the cells to pass this narrow zone is exceedingly short, so that the enzymes set free are not damaged, as can be proved by repeated grinding. It is a well-known fact

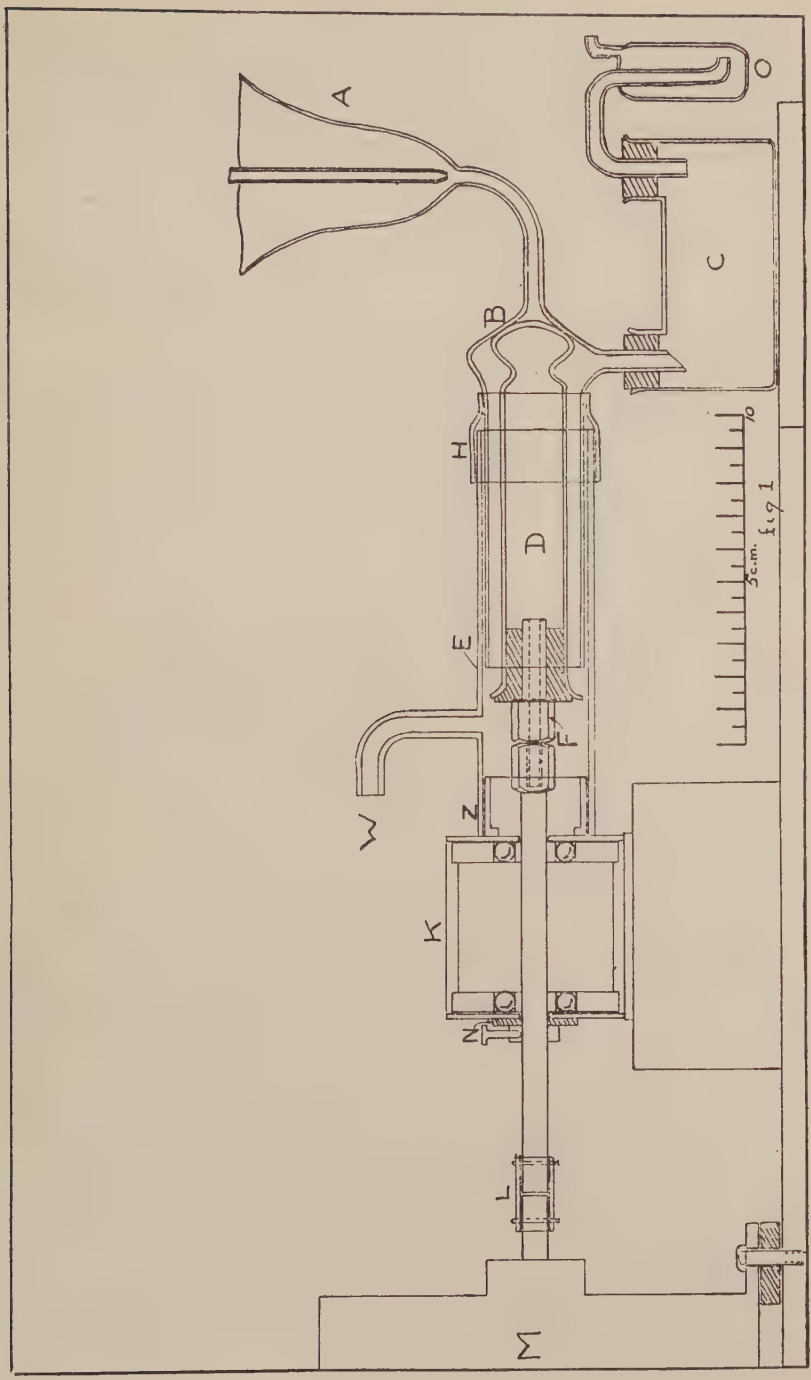

Fig. 1. Detail of figure 2: mounting of pestle and mortar.

that the grinding of agar or gelatine solutions for a longer period leads to the inability to solidify. But here in a single operation more than 90% of the cells are crushed and emptied, so that repeated grinding is as a rule superfluous.

Description of the mill.

As in the mill of KALNITSKY et al., the pestle is connected via a transmission to a motor M of about 1/10 H.P., mounted on rubber discs. The transmission is made in such a way, that shaft F with pestle D rotates 120-180 times per minute.

Between the shaft which carries the pestle and the motor axis there is a flexible connection L of rubber tubing. In order to avoid any strain on the pestle and to secure its smooth running, the shaft is adjusted with ball bearings K and carries at its right end a fixed nut in which the bolt F fits. Thread of bolt and nut must be such that rotation of the motor cannot lead to disconnecting of shaft and pestle.

The glass pestle D as well as the other part of the mill are made of Pyrex glass with a wall thickness of about 2 mm.

A close fitting rubber stopper is forced into the neck of the pestle. This rubber stopper carries the bolt F. To facilitate the pushing in of the rubber stopper this bolt has a hollow shaft.

The pestle is filled with water, an air-bubble being left in. Owing to the increase in weight the water promotes smooth running of the pestle without vibrations and prevents damage of the crushing surfaces at B. The pestle should turn without any oscillations. It can be detached for cleaning purposes etc., by loosening the bolt F. The stopper can be left in the pestle.

To the casing of the ball bearings K a copper cylinder E is screwed which carries the inlet tube W for nitrogen or any other inert gas. The screw-thread at Z should be air-tight; the rubber disc N closes the ball bearing casing, thus preventing gas leakage.

The cone-shaped mortar B ends in a glass cylinder, which closely fits in the copper cylinder E. The air-tight and flexible connection between E and B is a piece of rubber tubing H, for which we used a piece of ordinary bicycle tyre. The receiving flask C has a gas trap O, which is partly filled with water.

The mortar B is pressed on the pestle by a spring R (fig. 2). T is a rubber stopper (cut in two halves) fixed in a clamp. The spring R carries a screw with wingnut S. The clamp

which holds T must be fixed in such a position that, if wingnut S is loosened, the distance between the crushing surfaces amounts to 1—2 mm. With the wingnut S the force which spring R exerts can be regulated and herewith the speed of crushing. As the gas pressure in the container A is controlled by the pressure regulator

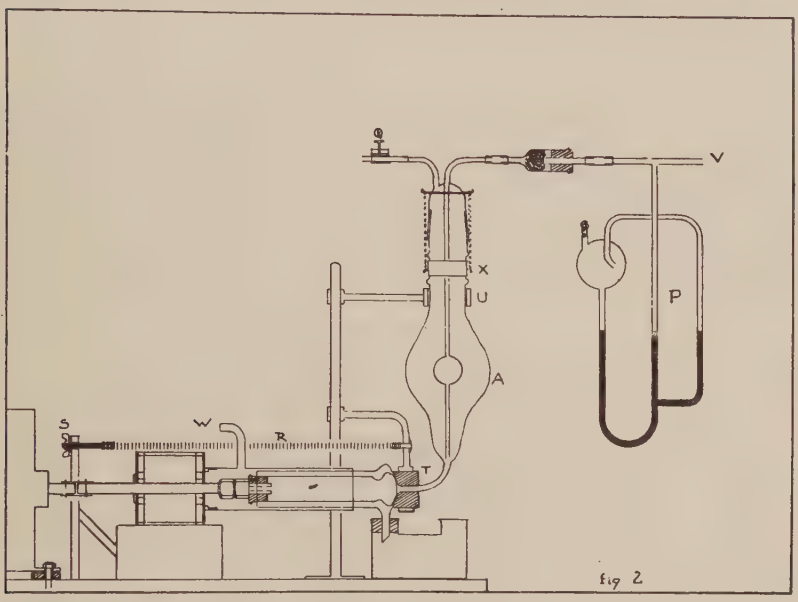

Fig. 2. General view of the instrument.

P (design of CHAMBERLAIN and DAVIES (5)), this speed depends only on the force exerted by spring R. The pressure regulator P is a safety-valve for pressures of e.g. 200 mm of mercury in gauge, the pressure we used in our experiments. The container A is kept loosely in position by clamp U.

Important is the shape of the crushing surfaces. The cone-shaped mortar and the spherical pestle have ground surfaces. If these surfaces touch each other along a broad zone, the resistance to the passage of fluid will be large. Consequently the distance between the two surfaces will have to be increased in order to obtain the passage of 1 drop of crushed bacteria suspension per 15 - 20 seconds — which we take as the normal output of the instrument — and it will appear that this distance will very soon surpass the dimensions of the bacteria, in which case these bacteria will pass the instrument without being crushed. It is of course essential that

the distance mentioned above does not surpass the dimensions of the bacteria which can only be achieved by taking care that the surface of contact between the moving glass parts is only a fine circular line. For ascertaining whether the surfaces touch each other along this very narrow zone, as should be the case, the following test is made.

Adjustment of the instrument.

Container A is partly filled with water and an air-pressure of 200 mm of mercury in gauge P is applied. With full tension of spring R, there should be practically no leaking of water at B (the pestle not running). In case of some leaking a small amount of fine carborundum powder in water is brought into the mortar and the motor is run for a few seconds. After thorough cleaning this test is repeated. As a rule the mill will crush satisfactorily. Dry running or running with water alone leads to immediate destruction of the ground glass surfaces. Minor damages of the crushing surfaces may be easily restored with a small amount of fine carborundum powder in water brought into the mortar, after which the pestle is run for a couple of seconds. There is practically no wear of the Pyrex ground surfaces, when creamy bacteria or yeasts suspensions are crushed. A ratio of wet bacteria to water of 1 : 1 gives good results.

Starting the instrument for crushing.

If the apparatus is adjusted correctly, parts A en D and the cap are disconnected by loosening S, T und U. These parts are now cleaned and if wanted sterilised in alcohol-HCl and rinsed with sterile water. After re-adjusting the apparatus, wingnut S is turned on to full tension, after which the bacteria suspension is poured into A. Air-pressure of 200 mm is then applied. In this position there should be no leaking at all through the crushing zone. Hosecock Q is loosened till an air stream bubbles through the suspension at a rate of about 5 bubbles per second. This prevents sedimentation. There should be no excessive foaming in the container A. The cap of container A is kept in place with the aid of two springs attached to X, a copper strip with two hooks. In the pressure regulator P too gas should always bubble through the right hand tube to prevent the flowing back of the bacteria suspension from A to P.

Now rubber stopper T is slightly withdrawn for a moment by hand in order just to moisten the grinding surface B with the suspension. This is necessary to prevent damage by dry running. Then the motor is started and the wingnut S is immediately loosened so far that the bacteria suspension passes at a rate of 1 drop per 20 seconds into the receiver C. If the suspension of the organisms is fairly homogeneous, this rate will remain the same, even for hours.

Care should be taken that the suspension does not contain any small particles of either sand or rubber. The latter, originating from the use of rubber stoppers in preparing the suspension, frequently occur and are not visible in the thick suspension. These very small rubber particles are trapped in the crushing zone, with the result, that the suspension all at once flows into the receiving flask. Therefore use of rubber stoppers should be avoided. Small cotton fibres are harmless in this respect.

Suspensions of bacteria, containing large amounts of polysaccharides e.g. dextran, are not satisfactorily crushed in this type of mill, because slime substances block the passage.

After the suspension has passed, the cell débris and undamaged cells are removed by centrifuging at 4.000 r. per min. during 40 minutes. The supernatant clear and somewhat viscous liquid is taken off with a pipette or decanted. If centrifugation under anaerobic conditions is required, the receiver C can be replaced by a centrifuge tube covered with a cap provided with in- and outlet. For strict anaerobic conditions thioglycollate or other reducing substances can be added.

The advantages of the instrument are:

a. No contact between bacteria suspension or enzyme solutions and metal or rubber surfaces.
b. No rise of temperature of the crushing surfaces even during long runs.
c. Crushing requires only one operation; repeated milling is not necessary and should be avoided as this might result in mechanical destruction of protein structures and inactivation of enzymes. The time of contact between bacteria and crushing surfaces is minimal.
d. No use is made of glass powder or other adsorbing crushing-aids.

e. Crushing in inert gas, in order to avoid irreversible oxidation of essential compounds, is easily done.
f. All glass parts can be easily cleaned and sterilised. Crushing under aseptical conditions is possible; the instrument can be used for the preparation of endotoxins from pathogens.
g. The amount of bacteria suspension to be crushed is practically unlimited. Small amounts of 2—3 ml are as easily crushed as large amounts. The time necessary for crushing 10 ml of a yeast or bacteria suspension varies from 30—130 minutes, depending on the shape of the mortar. In case large volumes have to be handled the receiving flask should be cooled in ice water, to prevent autolytic degradation of essential components in the enzyme suspension.
h. The apparatus is selfoperating and supervision is hardly necessary.

A skilfull instrument maker can make the whole apparatus in a few days. The total cost of the instrument is modest.

The mill was constructed at the Laboratory of Microbiology of the Technical University at Delft, by Mr P. WENTINK, whose help and advice is gratefully acknowledged.

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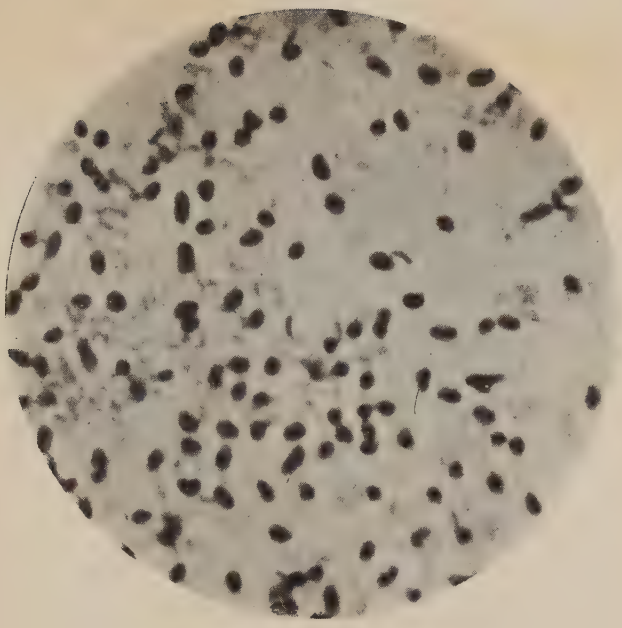

Fig. 1. *Ruminobacter parvum;* strain 1; 3 days old; purified culture. Hucker's methyl violet stain. 2500 ×.

Fig. 2. *Ruminococcus flavefaciens* on fibre; strain G; 8 days old; purified culture. Hucker's methyl violet stain. 1000 ×.

Fig. 3. *Ruminococcus flavefaciens* partly on fibre remnants; strain G; some specimens showing capsules; 6 days old; pure culture. Hucker's methyl violet stain. 1000 ×.

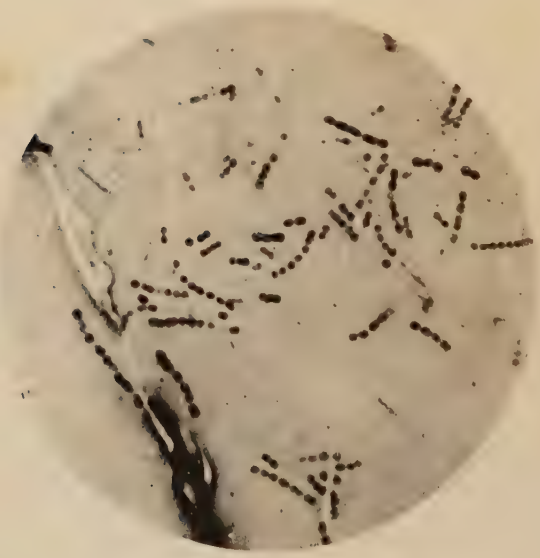

Fig. 4. Large type of cellulose-decomposing streptococcus partly on fibre remnants; 10 days old; enrichment culture. Gram stain. 1000 ×.